Thyroid Peroxidase Gene Expression Is Induced by Lipopolysaccharide Involving Nuclear Factor (NF)-κB p65 Subunit Phosphorylation

Magalí Nazar, Juan Pablo Nicola,* María Laura Vélez,* Claudia Gabriela Pellizas, and Ana María Masini-Repiso

Centro de Investigaciones en Bioquímica Clínica e Inmunología, Consejo Nacional de Investigaciones Científicas y Técnicas, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina

Thyroid peroxidase (TPO), a tissue-specific enzyme expressed in differentiated thyroid follicular cells, is a major antigen that has been linked to autoimmune thyroid disease. We have previously reported the functional expression of the lipopolysaccharide (LPS) receptor Toll-like receptor 4 on thyroid follicular cells. Here we investigated the effect of LPS in TPO expression and analyzed the mechanisms involved. We found a dose-dependent enhancement of TSH-induced TPO expression in response to LPS stimulation. EMSAs demonstrated that LPS treatment increased thyroid transcription factor-1 and -2 binding to the B and Z regions of TPO promoter, respectively. Moreover, LPS increased TSH-stimulated TPO promoter activity. Using bioinformatic analysis, we identified a conserved binding site for transcription nuclear factor-κB (NF-κB) in the TPO promoter. Chemical inhibition of NF-κB signaling and site-directed mutagenesis of the identified κB-cis-acting element abolished LPS stimulation. Furthermore, chromatin immunoprecipitation assays confirmed that *TPO* constitutes a novel NF-κB p65 subunit target gene in response to LPS. Additionally, our results indicate that p65 phosphorylation of serine 536 constitutes an essential step in the p65-dependent, LPS-induced transcriptional expression of *TPO*. In conclusion, here we demonstrated that LPS increases TPO expression, suggesting a novel mechanism involved in the regulation of a major thyroid autoantigen. Our results provide new insights into the potential effects of infectious processes on thyroid homeostasis. **(*Endocrinology* 153: 6114–6125, 2012)**

Thyroid peroxidase (TPO) is a thyroid-specific, 103-kDa glycosylated hemoprotein localized on the apical membrane of thyroid follicular cells (1). TPO catalyzes covalent incorporation of oxidized iodine into tyrosines in thyroglobulin and coupling of iodinated tyrosines to form thyroid hormones (2, 3). In addition, TPO has been identified as the major antigen constituent of the microsomal antigen involved in autoimmune thyroid diseases (4, 5). *TPO* gene expression is mainly regulated at the transcriptional level (6) and the pituitary hormone TSH constitutes the main hormonal regulator of *TPO* gene expression (7–9). The TPO promoter contains several binding sites for thyroid transcription factor (TTF)-1 (also named Nkx2.1), called A, B, and C; one site for TTF-2 (also known as FOXE1) called Z; one for nuclear factor 1 overlapping with TTF-1 site B; and one for paired box transcription factor 8 (Pax8) overlapping with TTF-1 site C (10–12).

Toll-like receptors (TLRs) constitute a family of receptors that recognize evolutionarily conserved pathogen-associated molecular patterns, playing important roles in host defense mechanisms (13). TLR4 in particular functions as the signaling component of the bacterial endotoxin lipopolysaccharide (LPS) recognition complex (14,

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* J.P.N. and M.L.V. contributed equally to this work.

Abbreviations: ChIP, Chromatin immunoprecipitation; dbcAMP, dibutyryl cAMP; FRTL-5, fisher rat thyroid cell line 5; IP, immunoprecipitation; LPS, lipopolysaccharide; Luc, luciferase; NF-κB, nuclear factor-κB; NIS, Na^+/I^- symporter; Pax8, paired box transcription factor 8; PKA, cAMP-dependent protein kinase; qPCR, quantitative PCR; S536, serine 536; SDS, sodium dodecyl sulfate; TPO, thyroid peroxidase; TLR, toll-like receptor; TTF, thyroid transcription factor.

15). LPS is an integral component of the outer surface of all Gram-negative bacteria exhibiting a wide array of biological effects, mainly on innate immune cells (16). However, growing evidence demonstrates LPS effects on endocrine cell homeostasis (17–21). Recently we reported that the endotoxin exerts a direct effect on thyroid cells by up-regulating TSH-stimulated thyroglobulin and Na^+/I^- symporter (NIS) gene expression (22, 23).

TLR recognition of microbial pathogens triggers conserved proinflammatory signaling pathways culminating in the activation of nuclear factor-κB (NF-κB) (24). TLR4-induced NF-κB activation plays a critical role in the regulation of several immune, inflammatory, and carcinogenic processes (25, 26). NF-κB belongs to the Rel-homology domain-containing protein family, which includes RelA/p65, p50, p52, c-Rel, and RelB, p65 being the most explored subunit (26–28). Each NF-κB constituent contributes to the structure of different homo- or heterodimers, thus conferring a degree of target gene specificity. We recently demonstrated the involvement of NF-κB in LPS-induced *NIS* transcriptional expression (29).

Considering the important role of TPO in autoimmune thyroid diseases, the aim of this work was to evaluate the effect of LPS on TPO expression in thyroid cells. Here we demonstrate that LPS enhances TSH-stimulated TPO expression. The effect of LPS was observed in TSH- or cAMP-stimulated cells, suggesting a possible cross talk between the pathways activated by LPS and TSH/cAMP. Interestingly, a bioinformatic analysis of the TPO promoter revealed a novel NF-κB binding site. Disruption of the identified cis-acting element as well as chemical inhibition of the NF-κB pathway abrogated LPS-induced TPO expression. Furthermore, using chromatin immunoprecipitation analysis, we corroborated that *TPO* is a novel target gene for p65 transactivation in response to LPS. These findings demonstrate the involvement of transcription factor p65 in LPS-stimulated *TPO* gene expression, supporting a novel model for LPS-induced gene expression in thyroid cells.

Materials and Methods

Reagents and antibodies

Phenol-extracted lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, monophosphoryl lipid A (Rd mutant) from *E. coli* F583, dibutyryl-cAMP, SB202190, PD98059, and BAY 11-7082 were from Sigma-Aldrich (St. Louis, MO). H89 was from Calbiochem (La Jolla, CA). Bovine TSH was a generous gift from the National Institute of Diabetes and Digestive and Kidney Diseases, National Pituitary Hormone Program and Dr. A.F. Parlow, National Institutes of Health (Torrance, CA).

Rabbit polyclonal antirat TPO antibody was as described (2). Monoclonal antibodies antihuman TPO, antihuman p65, and antihuman histone H1 and polyclonal antibodies antimouse TLR4, antihuman TTF-1, and antihuman TTF-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies antihuman phospho-p38 MAPK (Thr180/Tyr182), phospho-p44/42 MAPK, and phospho-p65 (Ser536) were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies antimouse α-tubulin and antimouse β-actin as well as nonspecific mouse IgG were from Sigma-Aldrich.

Promoter constructs

The pTPO Luc reporter plasmid containing the minimal rat TPO promoter (−429 to +3 bp) linked to luciferase (Luc), and constructs containing the TPO promoter mutated in the TTF-1 (pTPO Am), the TTF-1 and NF-1 (pTPO Bm), the TTF-2 (pTPO Zm), or the TTF-1/Pax8 binding consensus sites (pTPO Cm) were as previously described (6). TPO promoter deletions pTPO BZC (−180 to +3 bp), pTPO ZC (−152 to +3 bp), and pTPO C (−120 to +3 bp) were as reported (7, 12), considering +1 as the first nucleotide at which transcription starts.

The κB-site-deleted plasmid, pTPO ΔκB, (−228 to +139 bp) was generated by PCR and cloned into pGL3-basic vector (Promega, Madison, WI). The κB-site-mutated vector, pTPO κBm, was generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). pCMV-β-galactosidase was used to normalize transfection efficiency (Promega). The nucleotide sequence of all constructs was confirmed by DNA sequencing (Macrogen, Seoul, South Korea).

Cell culture and transient transfections

The thyroid cell line FRTL-5 was grown in DMEM/Ham F-12 medium supplemented with 5% (vol/vol) heat-inactivated calf bovine serum (Life Technologies, Inc., Langley, OK), 1 mIU/ml bovine TSH, 10 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 2 mM glutamine, and antibiotics (Sigma-Aldrich) (29). When cells reached 60–70% confluence, they were cultured for 5–6 d in the same medium but without TSH and containing 0.2% (vol/vol) calf serum (basal conditions). TSH-starved cells were treated with 10 or 100 ng/ml LPS in the absence or presence of 0.5 mIU/ml of TSH for different periods of time. When inhibitors were used, cells were preincubated with the inhibitor for 1 h before treatments. After the treatments, cell viability was higher than 95%, as determined by the Trypan blue dye exclusion assay.

FRTL-5 cells were transiently transfected in six-well plates using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) as described (29). After transfection, cells were split into 24-well plates at 80% of confluence. The following day cells were starved and treated as mentioned. After treatment, cells were harvested in passive lysis buffer (Promega) and assayed for Luc activity using a Luc reporter assay system (Promega). Luc activity was normalized relative to the levels of β-galactosidase activity (23).

RNA extraction and reverse transcription/quantitative PCR (qPCR)

Total RNA isolation and cDNA synthesis were performed as described (22, 30). qPCR analysis was carried out using an ABI Prism 7500 detection system (Applied Biosystems, Foster City, CA) and SYBR green chemistry as described (31). Gene-specific primer sets are reported in Supplemental Table 1, published on

The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method using β-actin as internal control. Specific target amplification was confirmed by automatic sequencing (Macrogen). All primers were from Sigma-Genosys (Houston, TX). Gene-specific primer sets and PCR efficiencies, calculated using dilution curves generated with cDNA or genomic DNA from FRTL-5 cells, are reported in Supplemental Table 1.

Protein extraction and Western blot

Total and nuclear protein extracts were obtained as described (23). To analyze phosphorylated proteins 1.5×10^6 cells were lysed in loading buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (Whatman, Clifton, NJ). Blots were incubated with 1 μg/ml anti-TPO, 2 μg/ml anti-TTF-1, 2 μg/ml anti-TTF-2, 1 μg/ml anti-p-p38, 1 μg/ml anti-p-p42/44, 1 μg/ml anti-p65 or 2 μg/ml anti-phospho-p65 (serine 536) antibodies overnight at 4 C. Similar loading was assessed by stripping and reprobing the same membrane with 0.5 μg/ml anti-β-actin, 0.2 μg/ml anti-α-tubulin, or 1 μg/ml anti-histone H1. Band intensities were evaluated densitometrically using ImageJ Software (National Institutes of Health, Bethesda, MD).

Electrophoretic mobility shift assay

Assays were performed as reported (23, 29). Synthesized double-stranded oligonucleotides (sequences in Supplemental Table 1) were labeled with [α^{32}P]-dATP using the Klenow fragment of DNA polymerase (New England BioLabs, Ipswich, MA). Nuclear extracts (5 μg) were incubated in binding mix buffer containing 40 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 5% glycerol, 200 μg/ml BSA, and 50 ng/μl of sonicated salmon sperm DNA on ice. A labeled oligonucleotide probe (1 ng DNA) was added, and incubation continued at room temperature for 30 min. The resulting DNA-protein complexes were separated from free labeled DNA on a 5% native polyacrylamide gel in 22 mM Tris (pH 8.5), 22 mM boric acid, and 0.5 mM EDTA and then vacuum dried and exposed to x-ray film. For competition assays, a 100-fold excess of cold oligonucleotide was added to the reaction mixture 30 min before addition of labeled oligonucleotide. Experiments analyzing complex specificity were performed incubating nuclear extracts with 0.5 μg monoclonal anti-p65 antibody for 1 h on ice before the labeled probe was added.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described (29). Briefly, after treatment, cross-linking was performed with 1% formaldehyde for 10 min and stopped in 125 mM glycine. Cells were washed in 1 mM phenylmethylsufonylfluoride-supplemented PBS. Nuclei were purified and resuspended in lysis buffer [50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and protease inhibitors]. Samples were sonicated to obtain 500-1000 bp chromatin fragments and then centrifuged at $10,000 \times g$. The supernatant was diluted 5-fold in immunoprecipitation (IP) dilution buffer [1% Triton X-100, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 150 mM NaCl] and precleared by adding salmon sperm DNA-saturated Protein A/G Plus Agarose (Santa Cruz Biotechnology). Precleared chromatin from 2×10^6 cells was incubated with 2 μg monoclonal anti-p65 antibody or control mouse IgG overnight. Immune complexes were purified with salmon sperm DNA-saturated Protein A/G Plus Agarose and washed four times with IP wash buffer [0.1% SDS, 1% Triton X-100, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 150 mM NaCl], twice with high-salt IP wash buffer [0.1% SDS, 1% Triton X-100, 5 mM EDTA, 500 mM NaCl, 50 mM Tris-HCl (pH 7.5)], and once with a buffer of 10 mM Tris-HCl (pH 8) and 1 mM EDTA. DNA was purified using Chelex-100 (Bio-Rad Laboratories, Hercules, CA). Eluted DNA was quantified by qPCR. The used primers are described in Supplemental Table 1. Relative fold of increase were calculated according to the following equation: fold change = $2^{-\Delta CT\,[CT\,(target\,gene)\,-\,CT\,(reference\,gene)\,-\,\Delta CT\,(control)]}$.

Statistical analysis

Results are presented as the mean ± SE from at least three independent experiments. Multiple group analysis was conducted by one-way ANOVA. As a posttest, the Student-Newman-Keuls multiple-comparisons test was used. Statistical tests were performed using GraphPad InStat 3 software (GraphPad Software, San Diego, CA). Differences were considered significant at $P < 0.05$.

Results

LPS stimulates TSH-induced TPO expression in thyroid cells

To investigate the effect of LPS on *TPO* gene expression, TSH-starved FRTL-5 cells were treated with LPS in the presence or absence of TSH for 48 h and TPO protein expression was evaluated. LPS treatment increased TSH-induced TPO expression in a dose-dependent manner. In contrast, LPS stimulation in the absence of TSH did not change TPO levels compared with those of nonstimulated cells (Fig. 1A). Consistent with this finding, LPS treatment for 12 h significantly increased TSH-stimulated TPO mRNA levels in a dose-dependent fashion (Fig. 1B). These results suggest that LPS modifies TSH-induced TPO expression, at least in part, at the transcriptional level.

LPS increases TSH-dependent TPO promoter activity

To study the mechanism involved in LPS-stimulated *TPO* transcription, we evaluated the transcriptional activity of the Luc reporter construct pTPO Luc in response to LPS in the presence or absence of TSH for 48 h in transiently transfected FRTL-5 cells. As reported, TSH increased TPO promoter activity (12). LPS induced a significant increase in TSH-mediated transcriptional activation of *TPO* (Fig. 2A). No effect of LPS was observed in the activity of the internal normalizer β-galactosidase.

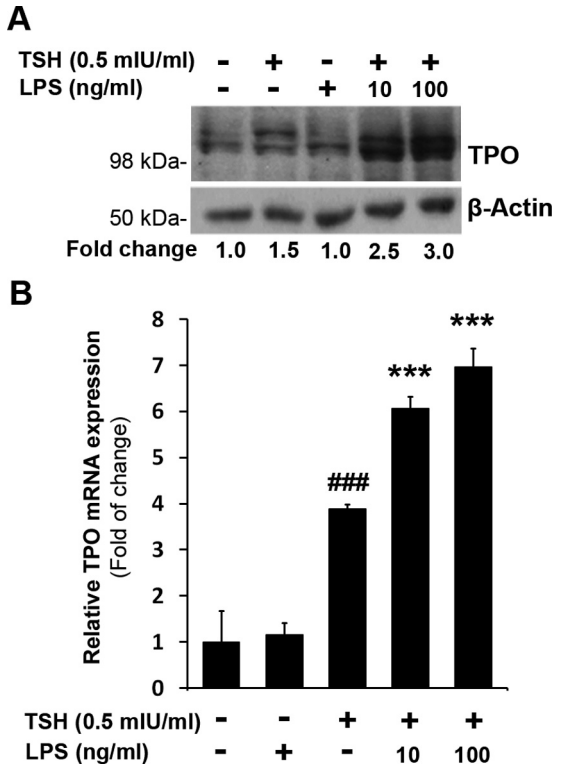

FIG. 1. LPS induces TSH-stimulated TPO expression. Starved FRTL-5 cells were treated with LPS (10–100 ng/ml) in the presence or absence of TSH (0.5 mIU/ml). A, Representative Western blot of whole-protein extracts obtained from FRTL-5 cells stimulated for 48 h. TPO expression was assessed by immunostaining with a monoclonal anti-TPO antibody. The same blot was reprobed with an anti-β-actin antibody as a loading control. Densitometric analysis was performed to determine the relative expression of TPO normalized to β-actin. Fold change indicates the mean of at least three independent experiments. B, Relative TPO mRNA quantification evaluated by RT/qPCR. Total RNA was extracted 12 h after treatment and reverse transcribed. TPO mRNA expression was relatively quantified to that of β-actin. Results are indicated as fold of change to the mRNA levels of untreated cells. ###, $P < 0.001$ *vs.* basal; ***, $P < 0.001$ *vs.* TSH (ANOVA; Student-Newman-Keuls).

B and Z cis-regulatory sites are involved in LPS-induced TSH-dependent *TPO* gene expression

FRTL-5 cells were transfected with serial 5′-deleted TPO promoter constructs linked to Luc to identify cis-acting elements involved in LPS-stimulated TPO expression (Fig. 2A, *left panel*). Although the site A-deleted construct (pTPO BZC) showed a significant response to TSH stimulation, we did not observe TSH-dependent LPS-stimulated TPO promoter activity (Fig. 2A). As described (12), the deletion of site B (pTPO ZC and pTPO C) abrogated TSH-dependent *TPO* transcriptional activity, thus blocking the LPS effect (Fig. 2A).

To explore the role of transcription factors involved in TSH-dependent, LPS-enhanced TPO expression, we analyzed pTPO Luc promoter mutated in several transcription factor binding sites (Fig. 2B, *left panel*). LPS increased the TSH-induced transcriptional activity of either site A or C mutants (pTPO Am and pTPO Cm, respectively) (Fig. 2B). Disruption of B or Z elements abolished TSH-induced TPO promoter activity (6, 12). Consequently, the activity of pTPO Bm or Zm in the presence of TSH was not modified by LPS. Thus, these results suggest that B and Z sites play a role in LPS-induced TPO promoter activity.

It is noteworthy that the deletion of the TPO promoter region comprised between −429 and −180 bp, which includes the TTF-1 binding site A, did not respond to LPS treatment. However, the mutant pTPO Am lacking the TTF-1 consensus site A was responsive to LPS, implying that additional unidentified cis-acting elements may be involved in TSH-dependent, LPS-enhanced *TPO* transcriptional expression.

LPS increases TTF-1 and TTF-2 binding to B and Z sites in the TPO promoter

By EMSA assays, we investigated changes induced by LPS in TTF-1 and TTF-2 binding to B and Z sites in the TPO promoter. Nuclear extracts from thyroid cells treated with TSH in the presence or absence of LPS were incubated with ^{32}P-labeled TPO-B or TPO-Z oligonucleotides. We observed a significant increase in the TSH-induced DNA-protein complex in LPS-treated cells (Supplemental Fig. 1, A and B, lanes 2 and 5). Binding specificity was assessed by displacement of the shifted band with an excess of the unlabeled self-oligonucleotide (TPO-B and TPO-Z cold) or by incubation with specific anti-TTF-1 or anti-TTF-2 antibodies (Supplemental Fig. 1, A and B, lanes 3 and 6, and lanes 4 and 7, respectively). Our results support the notion that in TSH-treated cells, LPS increases the binding of TTF-1 and TTF-2 to the B and Z sites, respectively.

To elucidate whether the augmented binding of TTF-1 and TTF-2 to their respective sites was due to an increase nuclear accumulation, we purified nuclear proteins from FRTL-5 cells stimulated with LPS in combination with TSH for 1 h. As shown in Supplemental Fig. 2, TTF-1 and TTF-2 nuclear levels were increased in LPS-treated cells. The increased nuclear translocation of the p65 subunit was used as a control, as we have previously reported (29). These results indicate that LPS regulates the binding activity of TTF-1 and TTF-2 to the TPO promoter, at least in part, by increasing the nuclear levels of these factors.

TLR4 mediates LPS-stimulated TPO expression

LPS responsiveness relies on the expression of functional TLR4 (15). In agreement, we reported TLR4 functional expression in thyroid cells (22). We analyzed the effect of LPS in the presence of a TLR4-specific blocking antibody. Preincubation with the anti-TLR4 antibody did not modify the TSH effect but abolished LPS-induced TPO promoter activity (Fig. 3, *upper panel*) and TPO protein

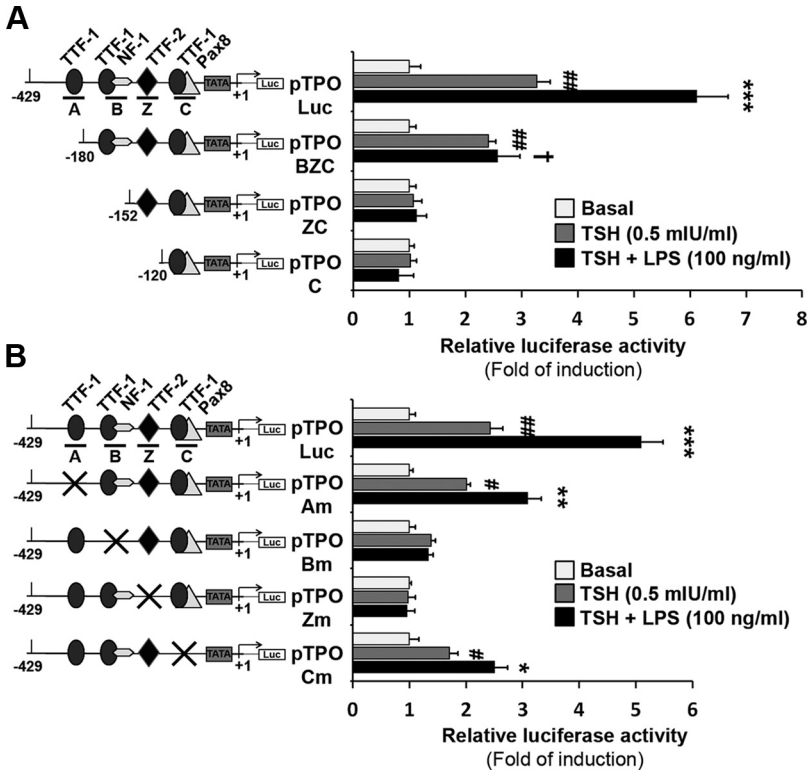

FIG. 2. LPS stimulates TSH-induced TPO promoter activity. A, FRTL-5 cells were transiently transfected with 5′-serial TPO promoter truncations linked to Luc. The *left panel* shows transfected constructs, and the *right panel* Luc activity in response to the indicated treatment. Starved cells were treated with LPS (100 ng/ml) in the presence of TSH (0.5 mIU/ml) for 48 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. ##, $P < 0.01$ *vs.* basal; ***, $P < 0.001$ *vs.* TSH; †, $P < 0.001$ *vs.* TSH + LPS in pTPO Luc (ANOVA; Student-Newman-Keuls). B, FRTL-5 cells were transiently transfected with different site-directed TPO promoter mutants linked to Luc. The *left panel* shows transfected constructions in which mutated binding sites are crossed. The *right panel* shows transcriptional activity in response to the indicated treatment for 48 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. ##, $P < 0.01$, #, $P < 0.05$ *vs.* basal; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ *vs.* TSH (ANOVA; Student-Newman-Keuls).

expression (Fig. 3, *lower panel*). When cells were treated with a nonrelated goat IgG, the effect of LPS was unaffected. Additionally, the TLR4 agonist lipid A mimicked LPS-induced TPO expression (Fig. 3), highlighting the participation of TLR4 in the effect of LPS on *TPO* gene expression.

LPS stimulates cAMP-induced TPO expression

The effect of LPS on TPO expression observed only in TSH-stimulated thyroid cells suggests a possible cross talk between the signals induced by TSH and LPS. The TSH-induced cAMP/protein kinase A (PKA) pathway is a key mediator in thyroid differentiation and TPO expression (9, 32). To determine whether the effect of LPS requires cAMP generation, starved FRTL-5 cells were treated with the cAMP analog, dibutyryl cAMP (dbcAMP) in the presence of LPS for 48 h. As expected, dbcAMP increased the TPO promoter activity (Fig. 4, *upper panel*) and protein expression (Fig. 4, *lower panel*). LPS treatment increased dbcAMP-stimulated TPO promoter activity and TPO protein expression (Fig. 4). We further investigated whether the PKA pathway is required for TSH-dependent, LPS-induced TPO expression. Starved FRTL-5 cells were incubated with TSH and LPS in the presence of the PKA inhibitor H89 for 48 h. PKA inhibition partially decreased TSH-stimulated TPO expression and significantly reduced the LPS-induced TPO promoter activity and TPO protein expression (Fig. 4). Our results suggest a crucial role for the TSH-stimulated cAMP/PKA pathway in LPS-enhanced TPO expression.

MAPKs p38 and p42/44 mediate LPS-induced TPO expression

In the TLR4-dependent signal transduction triggered by LPS, the MyD88-dependent signaling leads to activation of MAPKs (33). We examined the time-dependent phosphorylation of MAPKs after treatment with LPS in the presence of TSH in thyroid cells. In contrast to the slight activation of p38 and p42/44 in TSH-treated cells, LPS stimulation strongly increased p38 and p42/44 phosphorylation in TSH-stimulated cells (Fig. 5A). Additionally, inhibition of the p38 pathway with SB202190 abrogated the LPS-induced TPO promoter activity (Fig. 5B, *upper panel*) and TPO protein levels (Fig. 5B, *lower panel*). The p42/44 inhibitor PD98059 (50 μM) reduced the LPS-stimulated TPO promoter activity (Fig. 5B, *upper panel*) and TPO expression (Fig. 5B, *lower panel*). No significant effect of SB202190 or PD98059 on TSH-stimulated TPO expression was evident (Fig. 5B). Our results indicate that the MAPKs p38 and p42/44 are involved in the stimulatory effect of LPS on TPO expression.

A novel NF-κB cis-acting regulatory element in the TPO promoter mediates LPS-induced TPO expression

Bioinformatic analysis of the rat TPO promoter performed with MatInspector software (Genomatix, Munich, Germany) uncovered a putative κB-binding site (5′-AGAAACTCCC-3′) between nucleotides −311 and

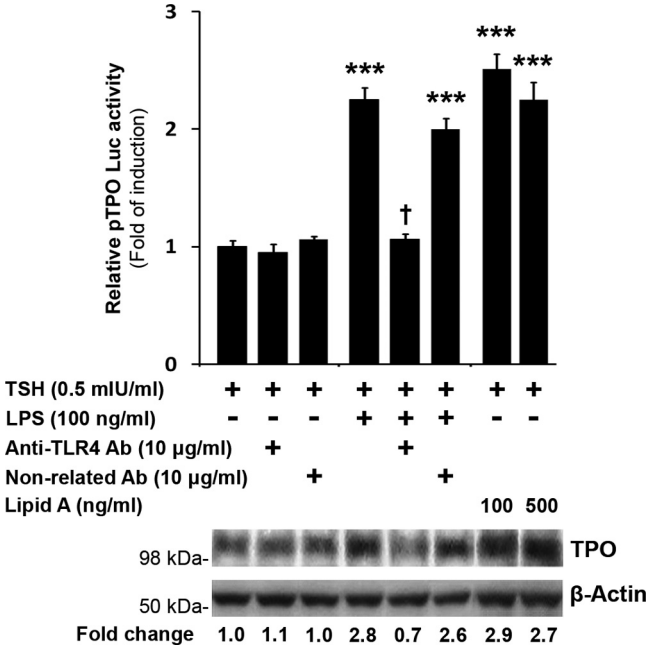

FIG. 3. TLR4 mediates LPS-stimulated TPO expression. *Upper panel*, Basal FRTL-5 cells transiently transfected with pTPO Luc promoter were stimulated with TSH (0.5 mIU/ml), TSH plus LPS (100 ng/ml), or the TLR4-agonist Lipid A (100 and 500 ng/ml) in the presence or absence of anti-TLR4 blocking antibody (10 μg/ml) for 48 h. Cells were incubated with an anti-TLR4 antibody or a nonrelated IgG as specificity control for 1 h before treatment. Results are expressed as Luc activity normalized to β-galactosidase and relative to the activity in nonstimulated cells. ***, $P < 0.001$ *vs.* TSH; †, $P < 0.001$ *vs.* same condition in absence of anti-TLR4 antibody (ANOVA; Student-Newman-Keuls). *Lower panel*, Representative Western blot analysis of whole-protein extracts from FRTL-5 cells stimulated as indicated for 48 h. TPO immunostaining was performed with a polyclonal anti-TPO antibody. β-Actin was used as loading control. Fold change indicates the mean of three independent experiments.

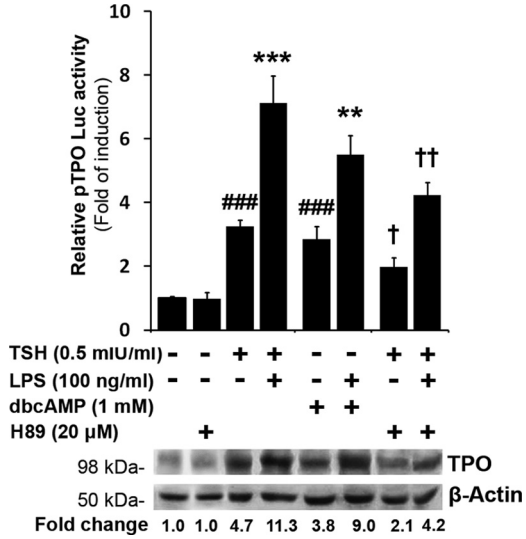

FIG. 4. LPS-enhanced TPO expression requires the cAMP/PKA pathway. *Upper panel*, Relative Luc activity of pTPO Luc transiently transfected FRTL-5 cells in response to the indicated treatments performed for 48 h. The PKA inhibitor H89 (20 μM) was added to the culture medium 1 h before treatment. Results are expressed as Luc activity normalized to β-galactosidase and relative to pTPO Luc activity in non-stimulated cells. ###, $P < 0.001$ *vs.* basal; ***, $P < 0.001$ *vs.* TSH; **, $P < 0.001$ *vs.* dbcAMP; †, $P < 0.01$, ††, $P < 0.005$ *vs.* same condition in the absence of inhibitor (ANOVA; Student-Newman-Keuls). *Lower panel*, Representative Western blot analysis of total protein extracts obtained from FRTL-5 cells stimulated for 48 h. TPO immunostaining was performed with a polyclonal anti-TPO antibody. Densitometric analysis was used to evaluate the relative increase of TPO expression normalized to β-actin. Fold change indicates the mean of three independent experiments.

−302 minus strand (core similarity = 1.0; matrix similarity = 0.858). The sequence alignment of TPO promoters from different species (human, mouse, and rat) showed strong conservation of the proposed κB-site (Fig. 6A), suggesting a potential trans-acting role of NF-κB in *TPO* transcriptional regulation.

We have recently reported that LPS treatment activates the NF-κB signaling pathway and showed the involvement of p65 as an intracellular mediator of LPS action in thyroid cells (29). Consequently, we analyzed the role of NF-κB in the LPS-induced TPO expression. The NF-κB inhibitor BAY 11-7082 blunted the effect of LPS in the TSH-stimulated TPO promoter activity (Fig. 6B, *upper panel*) and TPO protein expression (Fig. 6B, *lower panel*). In addition, different NF-κB inhibitors, such as sulfasalazine or Nα-tosyl-L-lysine chloromethyl ketone, showed similar results (data not shown). Interestingly, a significant reduction in TPO levels was observed in TSH-treated cells in the presence of BAY 11-7082 (Fig. 6B).

To investigate the role of NF-κB in the LPS-induced *TPO* transcriptional expression, we generated a deletion of the TPO promoter construct lacking the identified κB-site linked to Luc (pTPO ΔκB) (Fig. 6C). Although a decrease in TSH-induced activation of pTPO ΔκB compared with that of pTPO Luc was observed in transiently transfected cells, pTPO ΔκB did not respond to LPS treatment. Additionally, disruption by site-directed mutagenesis of the putative κB-site (pTPO κBm) abrogated TSH-dependent LPS responsiveness (Fig. 6C). The reduced TSH-induced stimulation of the TPO promoter suggests a physiological significance of NF-κB signaling in TSH responsiveness. Taken together, these results reveal the involvement of the NF-κB signaling pathway in the induction of *TPO* gene transcription triggered by LPS in thyroid follicular cells.

Experimental evidence supports a role for the p38 kinase in the modulation of NF-κB-induced gene transcription of several cytokines in immune cells (34, 35). Thus, we studied the potential relationship between activation of p38 and NF-κB signal pathways induced by LPS in thyroid cells. Starved cells treated with TSH plus LPS in the presence of the p38 inhibitor SB202190 revealed that p38 ac-

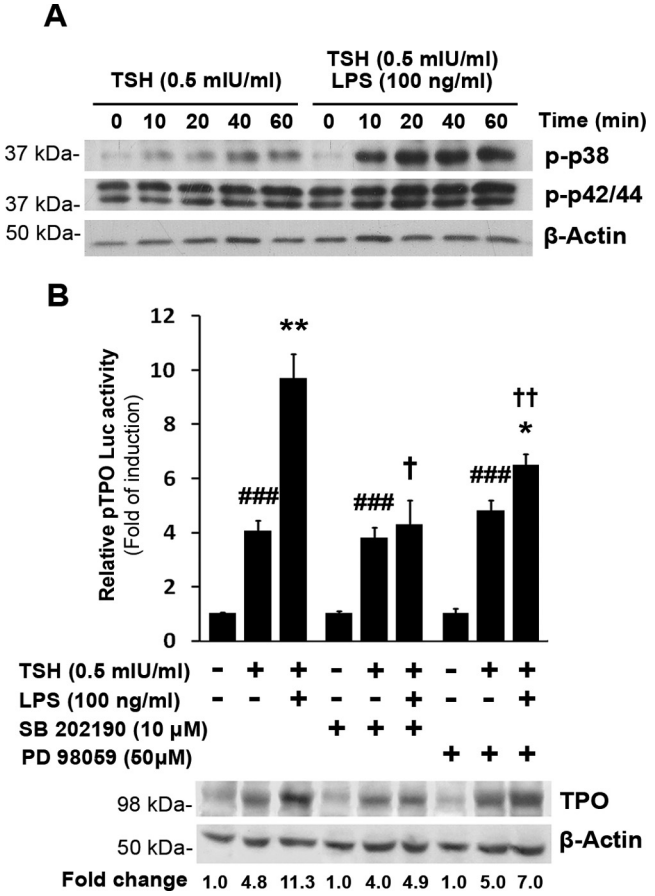

FIG. 5. MAPKs p38 and p42/44 are involved in LPS-stimulated TPO expression. A, Representative Western blot analysis of p38 and p42/44 phosphorylation status in response to the indicated treatments. Specific antibodies directed against specific phosphorylated epitopes of p38 (Thr180/Tyr182) and p42/44 (Thr202/Tyr204) were used. β-Actin was used as a loading control. B, *Upper panel*, FRTL-5 cells transiently transfected with pTPO Luc construct were stimulated as indicated for 48 h. Specific p38 and p42/44 inhibitors, SB202190 (10 μM) and PD98059 (50 μM), respectively, were added to the culture medium 1 h before treatment. Results are expressed as Luc activity normalized to β-galactosidase and relative to pTPO Luc basal activity. ###, $P < 0.001$ *vs.* basal; **, $P < 0.005$, *, $P < 0.01$ *vs.* TSH; †, $P < 0.001$, ††, $P < 0.005$ *vs.* same condition in the absence of inhibitor (ANOVA; Student-Newman-Keuls). *Lower panel,* Representative Western blot analysis performed on total lysates harvested from FRTL-5 cells stimulated as indicated for 48 h. TPO protein expression was evaluated with a polyclonal anti-TPO antibody. Fold change indicates the increase in TPO levels relative to β-actin expression used as a loading control.

tivation did not affect either the LPS-induced p65 nuclear translocation or the LPS-stimulated NF-κB transcriptional activity (Supplemental Fig. 3). These results suggest that nuclear translocation of NF-κB induced by LPS in thyroid cells may occur independently of p38 activation.

LPS induces p65 binding to the TPO promoter

Using EMSA, we studied the binding of FRTL-5 nuclear extracts to a TPO-κB radiolabeled oligonucleotide. As shown in Fig. 7A (lanes 1–3), a specific shifted band representing a NF-κB complex was induced by LPS treatment. Binding specificity was confirmed by competition assays using an excess of the unlabeled pTPO-κB probe (Fig. 7A, lanes 4–6). Additionally, we did not detect any shifted band when the κB core motif was mutated (TPO-κBm) (Fig. 7A, lanes 7–9). EMSA assay performed with an anti-p65 antibody showed a reduction in the complex intensity, suggesting the binding of p65 to the TPO-κB probe (Fig. 7A, lanes 10–12).

ChIP assays were performed to assess the *in vivo* interaction between p65 and the TPO promoter. Thyroid cells were stimulated with LPS in presence of TSH for 1 h. After cross-linking and sonication, soluble chromatin was immunoprecipitated using a monoclonal anti-p65 antibody. DNA contained in immunoprecipitates was analyzed by qPCR with specific primers designed to include the identified κB-site in the TPO promoter (P1) or an unrelated region (P2). Quantitative ChIP analysis revealed that LPS stimulation induced p65 binding to the sequence spanning the κB-site (Fig. 7B, *gray bars*) but not to an unrelated region of the TPO promoter (Fig. 7B, *black bars*). Nonspecific background was ruled out by performing the immunoprecipitation with a nonrelated mouse IgG (mock). These results demonstrate that, in response to LPS stimulation, p65 directly binds to the TPO promoter carrying a newly identified κB-consensus site.

p65 phosphorylation of serine 536 is essential to promote LPS-induced TPO expression

Once nuclear translocation is induced, the NF-κB complex undergoes different posttranslational modifications, thereby shaping the transcriptional responses (25, 26, 36). Within the posttranslational modifications, site-specific phosphorylation of p65 regulates its affinity for κB-sites, its oligomerization properties, and its interaction with transcriptional coactivators/repressors. Because phosphorylation of serine 536 (S536) positively influences p65 action in response to LPS (37, 38), we investigated the role of S536 phosphorylation in the LPS-induced TPO expression. Western blot analysis revealed that LPS treatment induced time-dependent p65 S536 phosphorylation in TSH-stimulated thyroid cells (Fig. 8A). Additionally, FRTL-5 cells were cotransfected with pTPO Luc together with an expression vector encoding human p65 in which serine 536 was replaced with alanine (p65 S536A) and costimulated with TSH and LPS for 48 h. The impaired S536 phosphorylation in the p65 S536A mutant protein prevented LPS-induced *TPO* transcriptional expression (Fig. 8B), indicating that the phosphorylation of S536 constitutes an essential step to induce p65-dependent transcriptional expression of *TPO* in response to LPS stimulation.

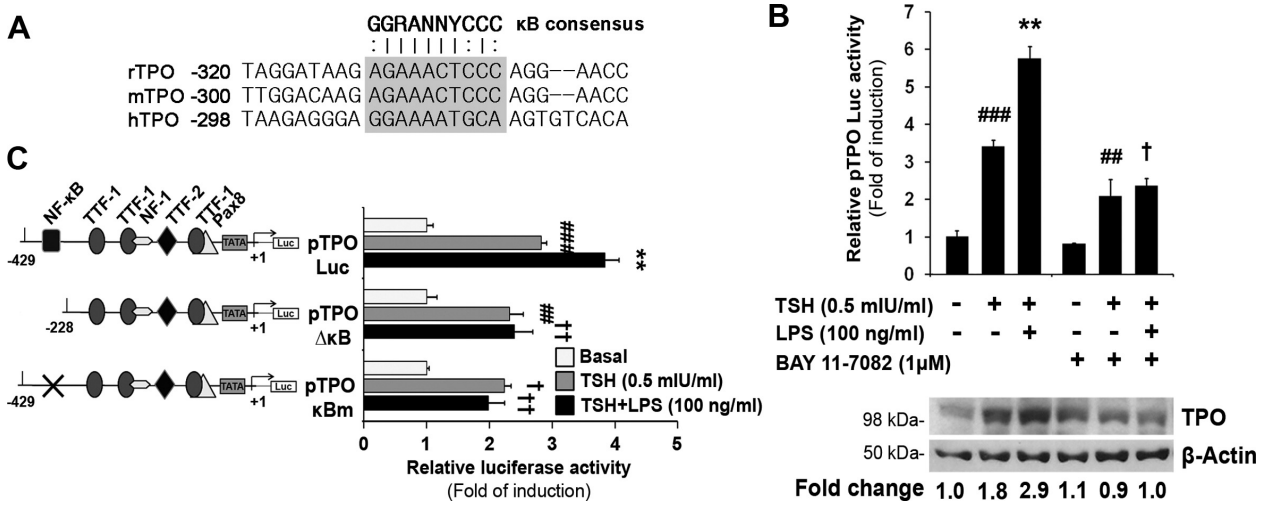

FIG. 6. A novel κB-consensus site identified in the TPO promoter is required for LPS action. A, Sequence alignment of TPO promoter sequences from human (hTPO), rat (rTPO), and mouse (mTPO) performed with Clustal W2 multiple sequence alignment (http://www.ebi.ac.uk/tools/clustalw2). *Shaded boxes* denote the identified putative κB-consensus site. Nucleotides are numbered considering +1 the first nucleotide in which the TPO transcription starts. B, *Upper panel*, FRTL-5 cells were transiently transfected with the TPO promoter construct pTPO Luc and stimulated after starvation as indicated for 48 h. The NF-κB inhibitor BAY 11-7082 (1 μM) was added to the culture medium 1 h before treatments. Results are expressed as Luc activity normalized to β-galactosidase and relative to pTPO Luc basal activity. ###, $P < 0.001$, ##, $P < 0.01$ *vs.* basal; **, $P < 0.01$ *vs.* TSH; †, $P < 0.01$ *vs.* same condition in the absence of inhibitor (ANOVA; Student-Newman-Keuls). *Lower panel*, Representative Western blot analysis showing TPO expression in response to the indicated treatments for 48 h. TPO expression was evaluated with a monoclonal anti-TPO antibody. Fold change indicates the increase in TPO levels relative to β-actin expression used as loading control. C, FRTL-5 cells were transiently transfected with the TPO promoter constructs shown in the *left panel*. The *right panel* shows transcriptional activity in response to the indicated stimulus for 48 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. ###, $P < 0.001$, ##, $P < 0.01$ *vs.* basal; **, $P < 0.01$ *vs.* TSH; †, $P < 0.05$ *vs.* TSH in pTPO Luc; ††, $P < 0.01$ *vs.* TSH + LPS in pTPO Luc (ANOVA; Student-Newman-Keuls).

Discussion

TPO is a thyroid-specific glycosylated hemoprotein located in the apical surface of the thyroid epithelium. In addition to its fundamental role in thyroid hormone biosynthesis, TPO has been identified as the major constituent of the microsomal antigen linked to autoimmune thyroid diseases (4, 5). Here we report the ability of the bacterial endotoxin LPS to enhance *TPO* gene expression in TSH-stimulated thyroid cells, reinforcing the previously proposed influence of LPS on thyroid differentiation genes (22, 23, 29). There are several reports on the ability of LPS to modify endocrine cell homeostasis. Hence, LPS increases GH expression and secretion in adenohypophysial cells (39), leptin production and release in adipose tissue (40), and cortisol secretion in human adrenocortical cells (17). LPS also suppresses testosterone biosynthesis in Leydig cells (41), whereas in pancreatic β-cells, LPS affects insulin content and secretion (18, 21). These findings denote that LPS released during an infectious process may alter the function of endocrine cells, including thyrocytes.

Although the thyroid gland is a common target of autoimmune diseases, the precise etiology that triggers thyroid autoimmunity remains largely unknown. Several studies have suggested autoimmune-prone genetic factors (42). In addition, environmental agents such as infections or excessive iodide intake have been correlated with the development of autoimmune thyroiditis (43, 44). Thus, thyroid dysfunction induced during viral infection could serve as a trigger for the development of thyroid autoimmunity (45, 46). Accumulating evidence demonstrates that TLRs are critical in various autoimmune diseases (47). A recent study regarding the development of autoimmune Hashimoto's thyroiditis in murine models demonstrated that bacterial LPS triggered experimental autoimmune thyroiditis in nonobese diabetic $H2^{h4}$ mice (48), supporting the involvement of TLRs in the development of thyroid autoimmunity.

Our results demonstrate a TSH-dependent increase of TPO mRNA and protein expression in response to LPS treatment in FRTL-5 cells. This effect seems to occur at the transcriptional level because TSH-dependent, LPS-stimulated TPO promoter activity was observed. When evaluated several serial 5′-deletions and site-directed mutants of the TPO promoter, deletion of the region −429 to −180 (pTPO BZC) blunted LPS-induced TPO expression, indicating the presence of crucial regulatory sequences related to LPS responsiveness. Interestingly, the cis-element A-mutated TPO promoter showed TSH-dependent LPS stimulation, suggesting that factors other than TTF-1 may be involved in the LPS effect. TPO promoter cis-regulatory

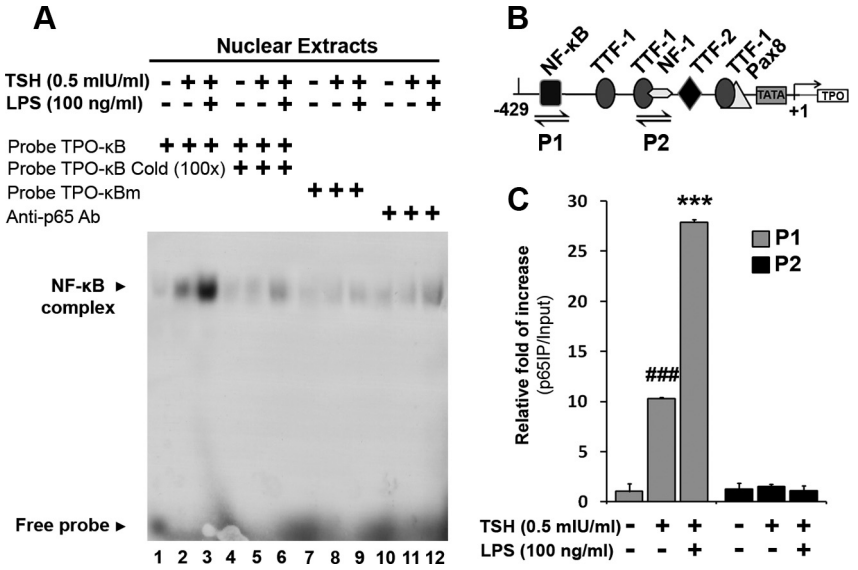

FIG. 7. LPS stimulates p65 binding to the NF-κB consensus site identified in the TPO promoter. A, Representative EMSA showing that LPS treatment increases the recruitment of the NF-κB complex to the region −318 to −294 within the TPO promoter region (TPO-κB probe) (lanes 1–3). Specificity was achieved by performing competition reactions in the presence of 100-fold excess of cold TPO-κB oligonucleotide (lanes 4–6) and by the significant reduction of shifted complex when the κB binding site is mutated (TPO-κBm) (lanes 7–9). Incubations performed in the presence of an anti-p65 antibody diminished the formation of specific NF-κB complex (lanes 10–12), thus denoting that p65 specifically recognizes the putative κB-cis-acting element identified in the TPO promoter region. B, Schematic representation of the rat TPO promoter. Positions are relative to the first nucleotide in which transcription starts, denoted as +1. The location of primer sets (P1 and P2) used in ChIP assays is shown. Transcription factor-binding sites are indicated. C, Basal FRTL-5 cells were treated under the indicated conditions for 1 h before cross-linking. DNA amount in immunoprecipitates was evaluated by qPCR. Results are expressed as relative fold of increase (p65 IP/input), arbitrarily considering the relation under basal condition as 1.0. Relative fold of increase was calculated according to the following equation: fold change $= 2^{-\Delta CT\ [CT\ (target\ gene)\ -\ CT\ (reference\ gene)]\ -\ \Delta CT\ (control)}$. ###, $P < 0.001$ *vs.* basal; ***, $P < 0.001$ *vs.* TSH (ANOVA; Student-Newman-Keuls).

regions B and Z, comprising binding sites for TTF-1 and TTF-2, respectively, are determinants for full TPO expression under TSH induction (6, 12). Accordingly, TPO promoter mutants unresponsive to TSH were insensitive to LPS stimulation. However, EMSA analysis demonstrated that LPS treatment increased TTF-1 and TTF-2 binding to the B and Z regions, respectively. These results indicate that the LPS-enhanced binding of thyroid-specific factors to the TPO promoter could be involved in the stimulated transactivation of the *TPO* gene.

TLRs play major roles in innate responses and in triggering adaptive immunity (49, 50). In accordance with a growing literature showing that TLRs exhibit functions in the homeostasis of several types of endocrine cells (17, 18, 21, 39–41), we reported a functional TLR4 expression conferring LPS responsiveness to thyroid follicular cells (22). Here we confirmed the involvement of TLR4 in LPS-induced changes in thyroid cells as demonstrated by blocking of LPS-stimulated TPO expression by specific anti-TLR4-neutralizing antibodies. In addition, we observed that the TLR4 agonist lipid A mimicked the LPS-induced stimulation of *TPO* transcriptional expression. Although these data support the idea that activation of TLR4-mediated signaling may influence thyroid cell homeostasis, we cannot rule out that thyroid-secreted proinflammatory cytokines stimulated by LPS might have an autoregulatory effect on thyroid physiology as suggested (51).

We previously reported that LPS lacks the ability to stimulate thyroid differentiation markers in TSH-starved cells (22, 23, 29), implying a cross talk between TSH-stimulated thyroid gene expression and the TLR4-dependent signal triggered by the endotoxin. It is well established that TPO expression in thyroid cells is dependent on TSH stimulation involving cAMP production because stimulators of the adenylate cyclase-cAMP system mimic TSH-induced TPO expression (8, 52). Here we studied the effect of LPS on cells treated with the cAMP analog dbcAMP. Although cell treatment with dbcAMP increased *TPO* transcription and protein expression, costimulation with LPS further increased TPO induction. Moreover, inhibition of cAMP-dependent PKA catalytic activity reduced the LPS effect on TPO expression, suggesting that LPS stimulation requires the integrity of the TSH-stimulated cAMP/PKA-mediated signaling pathway. Because cAMP-stimulated transcription is mediated by the PKA-induced p65 serine 276 phosphorylation in different cell types, the LPS-induced pathway might require TSH to exert its effect (53, 54).

Bioinformatic TPO promoter analysis uncovered a potential κB-consensus site comprised between nucleotides −311 and −302. Interestingly, the identified κB-site is highly conserved in rat, mouse, and human TPO promoters, suggesting a potential role for NF-κB as trans-acting factor related to TPO expression. Deletion or disruption by site-directed mutagenesis of the predicted κB cis-acting motif as well as chemical blockage of the NF-κB pathway inhibited the LPS effect, strongly indicating that NF-κB acts as a mediator in TPO expression induced by LPS. As observed, when the κB-site was mutated or NF-κB activation was blocked, TSH stimulation failed to fully activate TPO expression. In this regard, stimulating anti-TSH

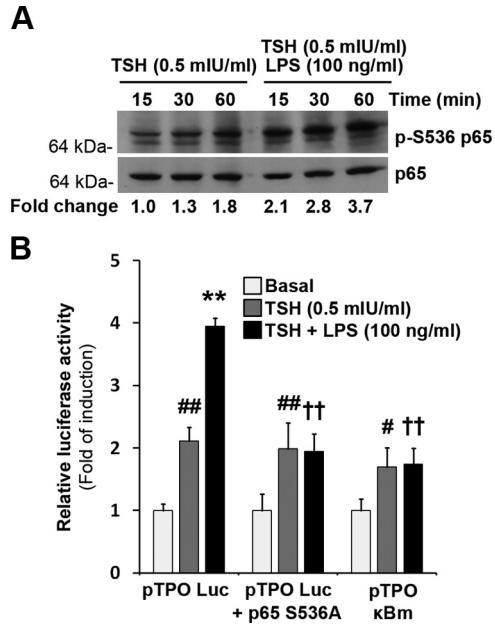

FIG. 8. S536 phosphorylation of p65 is a key step in LPS-induced TPO expression. A, Representative Western blot analysis showing S536 phosphorylated p65. Fold change indicates the relative increase of p65 phosphorylation considering total p65 protein expression as a loading control. Blots are representative of three independent experiments. B, FRTL-5 cells were cotransfected with pTPO Luc as reporter together with an expression vector encoding a p65 mutant in which serine 536 was replaced by alanine (p65 S536A). pTPO κBm was used as a non-LPS-responsive positive control. The same amounts of DNA were transfected under all the assayed conditions by adding pGL3-basic vector. Transfected FRTL-5 cells were starved and treated as indicated for 48 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. ##, $P < 0.01$, #, $P < 0.05$ *vs.* basal; **, $P < 0.01$ *vs.* TSH; ††, $P < 0.01$ *vs.* TSH + LPS in pTPO Luc (ANOVA; Student-Newman-Keuls).

receptor antibodies induce the NF-κB pathway (55), underscoring the potential ability of TSH to stimulate the NF-κB signaling pathway in thyroid cells. However, little is known about the role of NF-κB in thyroid physiology. We recently reported that disruption of the NF-κB binding site within the NIS promoter or p65 knockdown by small interfering RNA reduced TSH-induced NIS promoter activity in FRTL-5 cells (29), raising the question whether some members of the NF-κB family could be physiological trans-acting factors involved in TSH-dependent thyroid differentiation.

The NF-κB p65 subunit is a key transcriptional mediator in the TLR4-triggered LPS response (56, 57). We used different approaches to characterize the functional role of p65 as the mediator of the LPS effect on TPO expression. EMSA studies demonstrated that LPS treatment induced p65 binding to an oligonucleotide sequence containing the newly identified κB-site in the TPO promoter. We corroborated LPS-induced p65 binding to the TPO promoter *in vivo* using ChIP analysis. In accordance, we recently reported the participation of p65 in LPS-stimulated *NIS* transcriptional activity in FRTL-5 cells (29). All these data suggest a potential role for p65 as a mediator of the endotoxin action in thyroid cells.

Several kinases are involved in TLR4-mediated signaling pathways in response to LPS, including the activation of MAPKs and the transcription factor NF-κB (58, 59). Moreover, the NF-κB pathway regulates cytokine expression in response to proinflammatory stimuli requiring p38 activity (34). Assessment of p38 and p42/44 phosphorylation in response to LPS demonstrated a strong LPS-stimulated activation of p38 and p42/44 in thyroid cells. The TSH-induced phosphorylation of p38 and the activation of p42/44 have been reported (60, 61). Interestingly, inhibition of p38 activation blunted LPS-stimulatory action, whereas a partial inhibition was observed when p42/44 was repressed. We did not obtain evidence indicating a direct relationship between LPS-modulated p38 activation and nuclear translocation or transcriptional activity of NF-κB in thyroid cells. However, it has been suggested that p38 could modulate NF-κB-dependent gene transcription by modifying the interaction with coactivators (62) and the p38 requirement to promote NF-κB accessibility to potential cryptic sites by direct phosphorylation of nucleosome components (34, 63).

Under unstimulated conditions, NF-κB is sequestered in the cytoplasm associated with NF-κB-inhibitory proteins (26). When activated, NF-κB inhibitors are degraded and NF-κB subunits translocate to the nucleus, modulating a plethora of specific target genes (26). However, additional mechanisms as nuclear phosphorylation of the NF-κB p65 subunit are needed for full transactivation (25, 26, 36). Phosphorylation of p65 at serine 536 (S536) increased LPS-induced transcriptional activity in monocytes/macrophages (37). Interestingly, TLR activation induced NF-κB signaling in thyroid cells (29, 46). Accordingly, we observed that LPS treatment induced p65 S536 phosphorylation in FRTL-5 cells. Decreased *TPO* transcriptional expression in response to LPS treatment was observed when a mutant p65 carrying a serine to alanine substitution at position 536 was overexpressed in FRTL-5 cells, suggesting a key role of p65 S536 phosphorylation in LPS-induced *TPO* gene expression.

In summary, the present study reveals that thyroid follicular cells respond to LPS by increasing TSH-induced TPO expression. We uncovered a functional NF-κB binding site in the TPO promoter responsible for the LPS stimulatory effect. We also determined that LPS-mediated p65 S536 phosphorylation constitutes an essential step in NF-κB-induced *TPO* gene expression. Because TPO represents a major antigen associated with thyroid autoimmunity, our findings support a potential role for bacterial LPS

as a modulator of thyroid homeostasis entailing pathophysiological implications.

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Address all correspondence and requests for reprints to: Ana Maria Masini-Repiso, Ph.D., Centro de Investigaciones en Bioquímica Clínica e Inmunología, Consejo Nacional de Investigaciones Científicas y Técnicas, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, 5000 Córdoba, Argentina. E-mail: amasini@fcq.unc.edu.ar.

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Present address for J.P.N.: Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510.

Present address for M.L.V.: Centro de Investigación y Extensión Forestal Andino Patagónico, Departamento de Química, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, 9200 Esquel, Chubut, Argentina.

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