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# Nitric oxide-repressed Forkhead factor FoxE1 expression is involved in the inhibition of TSH-induced thyroid peroxidase levels

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ABSTRACT

Thyroid peroxidase (TPO) is essential for thyroid hormone synthesis mediating the covalent incorporation of iodine into tyrosine residues of thyroglobulin process known as organification. Thyroid-stimulating hormone (TSH) *via* cAMP signaling is the main hormonal regulator of TPO gene expression. In thyroid cells, TSH-stimulated nitric oxide (NO) production inhibits TSH-induced thyroid-specific gene expression, suggesting a potential autocrine role of NO in modulating thyroid function. Indeed, NO donors downregulate TSH-induced iodide accumulation and organification in thyroid cells. Here, using FRTL-5 thyroid cells as model, we obtained insights into the molecular mechanism underlying the inhibitory effects of NO on iodide organification. We demonstrated that NO donors inhibited TSH-stimulated TPO expression by inducing a cyclic guanosine monophosphate-dependent protein kinase-mediated transcriptional repression of the TPO gene. Moreover, we characterized the FoxE1 binding site Z as mediator of the NO-inhibited TPO expression. Mechanistically, we demonstrated that NO decreases TSH-induced FoxE1 expression, thus repressing the transcripcional activation of TPO gene. Taken together, we provide novel evidence reinforcing the inhibitory role of NO on thyroid cell function, an observation of potential pathophysiological relevance associated with human thyroid pathologies that come along with changes in the NO production.



## 1. Introduction

Thyroid peroxidase (TPO) is a thyroid-specific heme-containing glycoprotein localized on the apical membrane of thyroid follicular cells. TPO catalyzes iodide oxidation, covalent incorporation of iodine into tyrosine residues of thyroglobulin and coupling of iodinated tyrosines to form thyroid hormones (Ris-Stalpers and Bikker, 2010). The overall process of thyroid hormonogenesis is stimulated by the pituitary thyroid-stimulating hormone (TSH) acting through the G-protein coupled TSH receptor (TSHR) located on the basolateral membrane of thyroid cells (Colin et al., 2013). TSH activates the cAMP-protein kinase A (PKA) pathway, which is considered the main downstream mediator of TSH actions on thyroid cells (Muca and Vallar, 1994). TSH *via* cAMP signaling is the main hormonal regulator of TPO gene expression (Aza-Blanc et al., 1993; Gerard et al., 1988; Postiglione et al., 2002). However, TSHR signaling through other members of the G-protein family involving inositol phosphate/$Ca^{2+}$ pathway may also contribute to the regulation of TPO expression (Buch et al., 2008; Grasberger et al., 2007; Laugwitz et al., 1996).

TSH-regulated TPO expression is mainly stimulated at transcriptional level (Francis-Lang et al., 1992). The minimal TPO promoter required to confer TSH-responsiveness contains two binding sites for the thyroid transcription factor (TTF)-1 (also named Nkx-

*Abbreviations:* TPO, Thyroperoxidase; TSH, Thyroid Stimulating Hormone; cAMP, Cyclic Adenosine Monophosphate; NF-κB, Nuclear Factor-kappa B; NO, Nitric Oxide; NOS, NO synthase; cGMP, Cyclic Guanosine Monophosphate; cGC, Guanylate Cyclase; cGK, cGMP-dependent protein kinases; FRTL-5, Fisher rat thyroid cell line 5; SNP, Sodium Nitroprusside; GSNO, S-nitrosoglutathione; EMSA, Electromobility shift assay; ChIP, Chromatin Immunoprecipitation; qPCR, quantitative PCR; FSK, Forskolin; dbcAMP, dibutyryl-cAMP; PTIO, 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Rp, Rp-8-Br-PET-cGMPS.

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2.1), called B and C; one site for the Forkhead box protein E1 (FoxE1; formerly known as TTF-2) called Z; one for Nuclear Factor 1 (NF-1) overlapping with the TTF-1 binding site B, and one for the paired box domain transcription factor-8 (Pax8) that overlaps with TTF-1 site C (De Felice and Di Lauro, 2004; Ortiz et al., 1999). Beyond the minimal TSH responsive promoter, the TPO promoter contains the TTF-1 binding site A and a conserved binding site for the transcription factor NF-κB. The NF-κB binding site is required for the transcriptional expression of TPO in response to the bacterial endotoxin lipopolysaccharide (Nazar et al., 2012). FoxE1 is the main thyroid transcription factor that regulates TPO expression in response to TSH and insulin-like growth factor 1 (IGF-1) (Aza-Blanc et al., 1993). Moreover, FoxE1 expression itself is stimulated by TSH at transcriptional level (Ortiz et al., 1997). Therefore, FoxE1 binding site Z constitutes a hormone response element that regulates the expression of thyroid-restricted genes (Fernandez et al., 2013).

Nitric oxide (NO) is a ubiquitous signaling molecule involved in a wide variety of physiological processes. However, unbalanced production of NO has been associated with tumor progression and metastasis, and autoimmune diseases (Bogdan, 2001; Burke et al., 2013; Fukumura et al., 2006). Endogenous NO is generated from L-arginine by three NO-synthase (NOS) isoforms: neuronal (nNOS/NOS I), inducible (iNOS/NOS II) and endothelial (eNOS/NOS III), which are widely expressed (Burke et al., 2013). Particularly, NOS III is abundantly expressed in the thyroid follicular cells (Colin et al., 1997, 1995) and its expression is restricted to active thyroid follicles (Gerard et al., 2002). Most physiological NO actions are typically mediated through the soluble guanylate cyclase (sGC). NO activates sGC to produce the secondary messenger cyclic guanosine monophosphate (cGMP), thus acting through three main groups of cellular targets: cGMP-dependent protein kinases (cGK), cGMP-gated cation channels and cGMP-regulated phosphodiesterases (Francis et al., 2010; Martinez-Ruiz et al., 2011). Importantly, basal levels of endogenous NO-induced cGMP production have been reported in dog thyroid tissue (Esteves et al., 1992). Modifications of NOS expression are associated with several pathophysiological conditions of the thyroid gland (Colin et al., 1997, 1995; Patel et al., 2002; Sousa et al., 2010). In this regard, pro-inflammatory cytokines modulate NOS II expression and NO production in human thyrocytes (Kasai et al., 1995; van den Hove et al., 2002). Moreover, Gerard et al. (2006) reported a NO-mediated cytokine-induced reduction of the TSH-stimulated TPO and thyroid oxidase (ThOX) expression in human thyrocytes.

Most effects of NO on thyroid physiology have been demonstrated using NO donors. Several reports demonstrated that the classical NO donors sodium nitroprusside increases cGMP production in different thyroid cell models (Bazzara et al., 2007; Bocanera et al., 1997; Esteves et al., 1992; Millatt et al., 1993). Moreover, different studies have evidenced that NO donors downregulate TSH-induced iodide accumulation and organification in thyroid cells (Bazzara et al., 2007; Bocanera et al., 1997; Costamagna et al., 1998). Complementarily, we evaluated the role of endogenous NO production on thyroid cell function and differentiation using non-selective NOS inhibitors, concluding that endogenously produced NO acts as a negative regulator of TSH-stimulated gene expression and proliferation in thyrocytes (Fozzatti et al., 2007). Together, these results suggested a potential role of NO as an inhibitory autocrine feedback loop in the regulation of TSH-dependent thyroid cell function.

In the present study, we aimed to characterize the molecular mechanism underlying the inhibitory effects of NO on iodide organification studying the influence of NO donors on TPO gene expression in the thyroid cell line FRTL-5. We observed that different NO donors inhibited TSH-stimulated TPO protein expression involving a transcriptional repression of TPO gene. The inhibitory effect of NO occurs downstream of TSHR stimulation and involves the activation of cGK signaling. Furthermore, we provide evidence supporting that NO-modulated TPO transcriptional expression involves NO-triggered inhibition of FoxE1 expression, which reduces transactivation of the TSH-stimulated TPO promoter.

## 2. Materials and methods

### 2.1. Cell culture

The rat thyroid cell line FRTL-5 was grown in DMEM/Ham F-12 medium, supplemented with 5% heat-inactivated calf bovine serum (Life Technologies, Carlsbad, CA, USA), 1 mIU/ml bovine TSH (National Hormone and Peptide Program, Torrance, CA, USA), 10 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 2 μmol/ml glutamine and antibiotics (Sigma–Aldrich, Saint Louis, MO, USA) (Nicola et al., 2011). When cells reached 60–70% confluence, they were cultured in the same media except without TSH and containing 0.2% calf serum (basal media). Cells were maintained for 5–6 days in basal media, allowing cells to be quiescent before each experiment. TSH-starved cells were treated with the indicated concentrations of the classical NO donors sodium nitroprusside (Merck, Darmstadt, Germany) and S-nitrosoglutathione (Sigma–Aldrich) in the absence or presence of 0.5 mIU/ml TSH. To control specificity of NO effects, cells were pretreated with equimolar concentrations of the NO scavenger 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (Sigma–Aldrich) for 30 min before NO donors treatment. Forskolin (FSK), dibutyryl cyclic-adenosine monophosphate (dbcAMP), 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) KT5823 and Rp-8-Br-PET-cGMPS (Rp) were from Sigma–Aldrich.

### 2.2. Nitrite determination

The production of nitrite, a stable oxidation end product of NO metabolism, was measured in the culture media using Griess reagent as described (Bazzara et al., 2007). Results were expressed as nmol nitrite/μg DNA.

### 2.3. Western blot

Total protein extracts were prepared as described (Montesinos et al., 2012). Proteins (40 μg) were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California, USA). Membranes were blocked in 5% skimmed milk or 5% BSA, 0.1% Tween 20, Tris-buffered saline (TBS-Tween). Blots were incubated with primary antibodies in TBS-Tween overnight at 4 °C followed by incubation with HRP-conjugated secondary antibodies. Blots were striped and reprobed with anti-β-actin antibody to assess equal loading. The antibodies used were anti-TPO (sc-58432), anti-FoxE1 (sc-16392) and anti-β-actin (sc-8432) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Proteins were visualized by the enhanced chemiluminescence Western blot detection system (Amersham Life Sciences, Buckinghamshire, UK). Band intensities were measured densitometrically using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

### 2.4. Real-time PCR

Total RNA isolation, cDNA synthesis and quantitative PCR (qPCR) were performed as described (Nicola et al., 2012). Briefly, reactions were carried out using cDNA template (equivalent to 250 ng of total RNA), 0.6 μM primers, 2x Brilliant SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA, USA), and 30 nM Rox as passive reference

dye. Gene specific primer sets were as follow: TPO (139 bp) 5′-GCATGTATCATTGGGAAGCA (forward) and 5′-CGGTGTTGTCACA-GATGACC (reverse); FoxE1 (385 bp) 5′-AGAAGTGGCAGAACAGCATC (forward) and 5′-TAGGGAACCAAGCCGAAGAC (reverse); β-actin (138 bp) 5′-GGCACCACACTTTCTACAATG (forward) and 5′-TGGCTGGGGTGTTGAAGGT (reverse). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method normalized against the housekeeping gene β-actin. For each pair of primers a dissociation plot resulted in a single peak, indicating that only one cDNA specie was amplified. Specific target amplification was confirmed by automatic sequencing (Macrogen Inc., Seoul, South Korea). The efficiency of qPCR reactions for each pair of primers was calculated using standard curves generated by serial dilutions of cDNA or genomic DNA from TSH-stimulated FRTL-5 cells. All qPCR efficiencies ranged between 95 and 105% in different assays.

### 2.5. Plasmids

The pTPO reporter plasmid containing the minimal rat TPO promoter (−429 to −3 bp) linked to luciferase (Luc), and its site-directed mutants missing the NF-κB (pTPO κBm), TTF-1 and NF-1 (pTPO Bm) and FoxE1 (pTPO Zm) binding sites were as described (Francis-Lang et al., 1992; Nazar et al., 2012). The TPO promoter deletions pTPO BZC (−180 to −3 bp) and pTPO ZC (−152 to −3 bp), and the constructs containing the B and Z site (pTPO BZ) and 12 tandem repeats of the Z site from the TPO promoter (p12Z) linked to Luc were as reported (Aza-Blanc et al., 1993; Ortiz et al., 1999). The −2014 to +475 bp (+1 denotes the transcription start site) DNA fragment of the FoxE1 promoter was amplified by PCR using the following primers containing SmaI and KpnI restriction sites 5′-GGAGGGGTACCGCCACAGTCAAAGGAGC (forward) and 5′-GGA-GACCCGGGAGAGCAGGGGACTTCG (reverse), and cloned into the corresponding sites of the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). The normalization reporter pCMV-β-galactosidase was purchased from Promega. The expression vector encoding FoxE1 under transcriptional control of the cytomegalovirus promoter was as reported (Zannini et al., 1997). The nucleotide sequence of all constructs was confirmed by DNA sequencing (Macrogen Inc.).

### 2.6. Transient transfection and reporter gene assay

FRTL-5 cells were seeded into six-well plates at 50–70% confluence, and 24 h later, cells were transiently transfected with 2 μg of luciferase reporter-promoter constructs/well using Lipofectamine 2000 (Life Technologies) as described (Nicola et al., 2010). To evaluate promoter activity, cells were split into 24-well plates at 80% confluence the day after transfection. The next day, growth media was replaced by basal media and transfected cells were starved and treated as mentioned. The luciferase activity was measured using a Luciferase Assay System (Promega) according to manufacturer's instructions. To evaluate transfection efficiency, cells were cotransfected with 0.2 μg/well of the normalization vector encoding β-galactosidase. Luciferase activities were normalized relative to β-galactosidase activity values. When the effect of FoxE1 overexpression was tested, FRTL-5 cells were cotransfected with 1 μg of pTPO, 1 μg of FoxE1 expression vector, and 0.4 μg of β-galactosidase reporter per well of a six-well plate.

### 2.7. Electromobility shift assay (EMSA)

Assays were performed as previously reported (Montesinos et al., 2006; Velez et al., 2006). Synthesized double-stranded oligonucleotide Z (5′-ACAAATACTAAACAAACAGAATGG-3′) derived from the specific FoxE1 binding Z site of the TPO promoter was labeled with [α-$^{32}$P] dATP by using the Klenow fragment of DNA polymerase (New England BioLabs, Ipswich, MA, USA) and purified in a Sephadex G25 column (Sigma–Aldrich). 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 (Fraction V), and 50 ng/μl of sonicated salmon sperm DNA on ice. 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, 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. Supershift experiments were performed incubating nuclear extracts with 0.5 μg anti-FoxE1 antibody (sc-16392, Santa Cruz Biotechnology) for 1 h on ice before the labeled probe was added, and then processed as indicated above.

### 2.8. Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Mascanfroni et al., 2010). Briefly, cells were crosslinked in culture media containing 1% formaldehyde. Nuclei were purified and lysed in 50 mM Tris-HCl (pH 8), 10 mM EDTA, and 1% sodium dodecyl sulfate (SDS). Genomic DNA was broken by sonication and 10-fold diluted in IP Dilution Buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 0.5% Nonidet P-40]. Immunoprecipitation was performed with 2 μg of anti-FoxE1 antibody (sc-16392, Santa Cruz Biotechnology) or control mouse IgG. Immune complexes were purified with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed four times with IP Dilution Buffer containing 0.1% SDS, twice with High Salt IP Wash Buffer [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, 0.1% SDS and 1% Triton X-100], and once with TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA]. DNA was purified using Chelex-100 (Bio-Rad Laboratories). Immunoprecipitated DNA was quantified by qPCR using the following primer set that amplifies a region of 163 bp comprising BZ binding sites within the TPO promoter: 5′-CTGCTTTCTATGAGTGGCACC (forward), 5′-GGTAACCAAGTCTCCAGAGAG (reverse). Relative fold increase was calculated according to the equation: $2^{-[(Ct.input\ -\ Ct.target)\ -\ (Ct.input\ -\ Ct.mock)]}$ (Nicola et al., 2010).

### 2.9. Statistical analysis

Results are reported as the mean ± SD. Multiple group analysis was conducted by one-way ANOVA followed by Student–Newman–Keuls post-test. Statistical tests were performed using GraphPad InStat 3 software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at p-values $<0.05$.

## 3. Results

### 3.1. NO donors inhibit TSH-induced TPO expression

We have previously reported that the classical NO donor sodium nitroprusside (SNP) inhibits TSH-stimulated TPO mRNA expression (Bazzara et al., 2007). To investigate the molecular mechanism involved in the effect of NO on TPO gene expression, we first evaluated the effect of different NO donors on TSH-induced TPO protein expression in FRTL-5 cells. Thyroid cells were treated with the structurally unrelated NO donors SNP and S-nitrosoglutathione (GSNO) in the presence or absence of TSH for 24 h. As expected, SNP

**Table 1**
Nitrite accumulation after treatment of FRTL-5 with NO donors.

| | Nitrite (nmol/μg DNA) |
|---|---|
| **SNP** (μM) | |
| 0 | 0.06 ± 0.05 |
| 50 | 0.84 ± 0.32[a] |
| 100 | 1.19 ± 0.08[a] |
| 200 | 1.52 ± 0.17[a] |
| **GSNO** (μM) | |
| 0 | 0.09 ± 0.08 |
| 50 | 1.15 ± 0.34[a] |
| 100 | 1.66 ± 0.15[a] |
| 200 | 2.09 ± 0.28[a] |

[a] $p < 0.001$ vs TSH alone (without NO donors). Nitrite level was determined in culture medium as described. Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. NO: nitric oxide; SNP: Sodium nitroprusside; GSNO: S-Nitrosoglutathione.

and GSNO caused a dose-dependent NO release, as quantified by accumulation of nitrite, the major stable metabolite of NO degradation, in the culture medium of TSH-starved FRTL-5 cells (Table 1). Treatment of thyroid cells with SNP (100–300 μM) decreased TSH-induced TPO protein expression in a dose-dependent manner (Fig. 1A). Similar results were obtained with the physiological NO-donor GSNO (100–300 μM) (Fig. 1B). Both agents had no significant effect on TPO expression in the absence of TSH stimulation (Fig. 1A–B).

To further investigate the mechanism involved in the NO-reduced TPO protein expression, we evaluated TSH-stimulated TPO mRNA expression in response to NO donors. Incubation of FRTL-5 cells with SNP for 24 h led to a significant reduction of TPO mRNA as compared to control TSH-treated cells (Fig. 1C). In agreement, similar inhibition was observed in the presence of GSNO (Fig. 1D). Basal TPO mRNA levels were not modified by NO donors in the absence of TSH (data not shown). These results suggest that NO-repressed TSH-induced TPO expression involves, at least in part, a transcriptional inhibition of TPO gene.

### 3.2. NO donors downregulate TSH-dependent TPO gene transcriptional activity

To study NO-induced TPO gene transcriptional repression, we assessed the effect of NO donors on rat TPO promoter activity. The transcriptional activity of the luciferase (Luc) reporter construct pTPO transiently transfected into FRTL-5 cells was evaluated in response to NO donors in the presence or absence of TSH for 24 h. As reported (Ortiz et al., 1999), TSH stimulated TPO promoter activity. The NO donors SNP and GSNO inhibited the TSH-induced TPO promoter transcriptional activation (Fig. 2A). Basal TPO promoter activity was not affected by NO donors (data not shown).

To identify *cis*-acting elements involved in the transcriptional effect of NO donors on TPO repression, we analyzed the transcriptional activity of serial 5′-deleted TPO promoter constructs linked to Luc (Fig. 2A, left panel). Site NF-κB and A-deleted construct pTPO BZC showed a significant response to TSH stimulation, whereas NO donors decreased TSH-stimulated pTPO BZC promoter activity (Fig. 2A). Moreover, sites NF-κB, A and B-deleted reporter pTPO ZC abrogated TSH-dependent TPO transcriptional activity (Ortiz et al., 1999), thus masking the effect of NO donors (Fig. 2A).

To further explore the role of transcription factors involved in the effect of NO donors on TPO expression, we tested several TPO promoter constructs bearing point mutations in different transcription factor binding sites (Fig. 2B, left panel). We observed that NO donors inhibited the TSH-induced transcriptional activity of NF-κB mutant (pTPO κBm) (Fig. 2B). Moreover, we could not assess a direct effect of NO donors on TTF-1/NF-1 and FoxE1 binding sites as

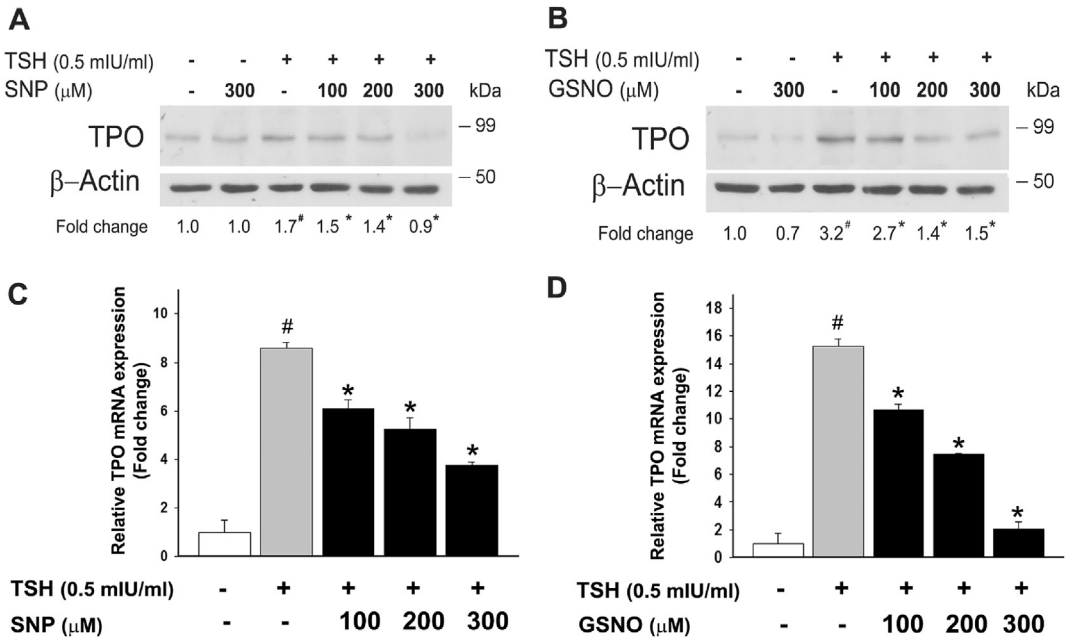


**Fig. 1. NO donors inhibit TSH-induced TPO expression.** Starved FRTL-5 cells were treated with SNP (100–300 μM) or GSNO (100–300 μM) in the presence or absence of TSH (0.5 mIU/ml) for 24 h. **A** and **B**, Representative Western blot of whole protein extracts evaluating TPO expression. β-actin expression was used as control for equal protein loading (*lower panel*). 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. #$p < 0.01$ vs Basal; *$p < 0.05$ vs TSH (ANOVA; Student–Newman–Keuls). **C** and **D**, Relative TPO mRNA quantification evaluated by RT/qPCR. TPO mRNA expression levels were quantified relative to those of β-Actin. Results are indicated as fold change relative to the mRNA levels of untreated cells. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #$p < 0.001$ vs Basal; *$p < 0.001$ vs TSH (ANOVA; Student–Newman–Keuls).

disruption of the B and Z elements abolished TSH-induced TPO promoter activity.

TSH-stimulated TPO expression is mainly regulated by FoxE1 binding to the *cis*-regulatory element Z. Mutagenesis of the binding site Z, impairing FoxE1 binding to the TPO promoter, abrogates the TSH-induced stimulatory effect (Fig. 2B). Furthermore, TPO promoter response to TSH is dependent on an active cooperation between FoxE1 and the constitutive transcription factor CAAT box-binding transcription factor nuclear factor-1 (CTF/NF-1) (Ortiz et al., 1999). Therefore, we evaluated the transcriptional activity of the TSH-responsive pBZ reporter containing a fragment of the TPO promoter comprising B and Z binding sites. As expected, the transcriptional activity of the pBZ was enhanced by TSH (Fig. 2C). Moreover, SNP and GSNO inhibited the effect of TSH on the pBZ transcriptional activity (Fig. 2C). In order to assess the role of the FoxE1 binding site Z in the effect of NO donors on TPO transcriptional activity, we evaluated the transcriptional activity of an artificial FoxE1 responsive vector containing twelve Z elements in tandem linked to luciferase (p12Z). As previously reported (Aza-Blanc et al., 1993), the FoxE1 responsive element p12Z is activated by TSH stimulation (Fig. 2C). Interestingly, NO donors inhibited the TSH-induced p12Z transcriptional activity. Taken together, these

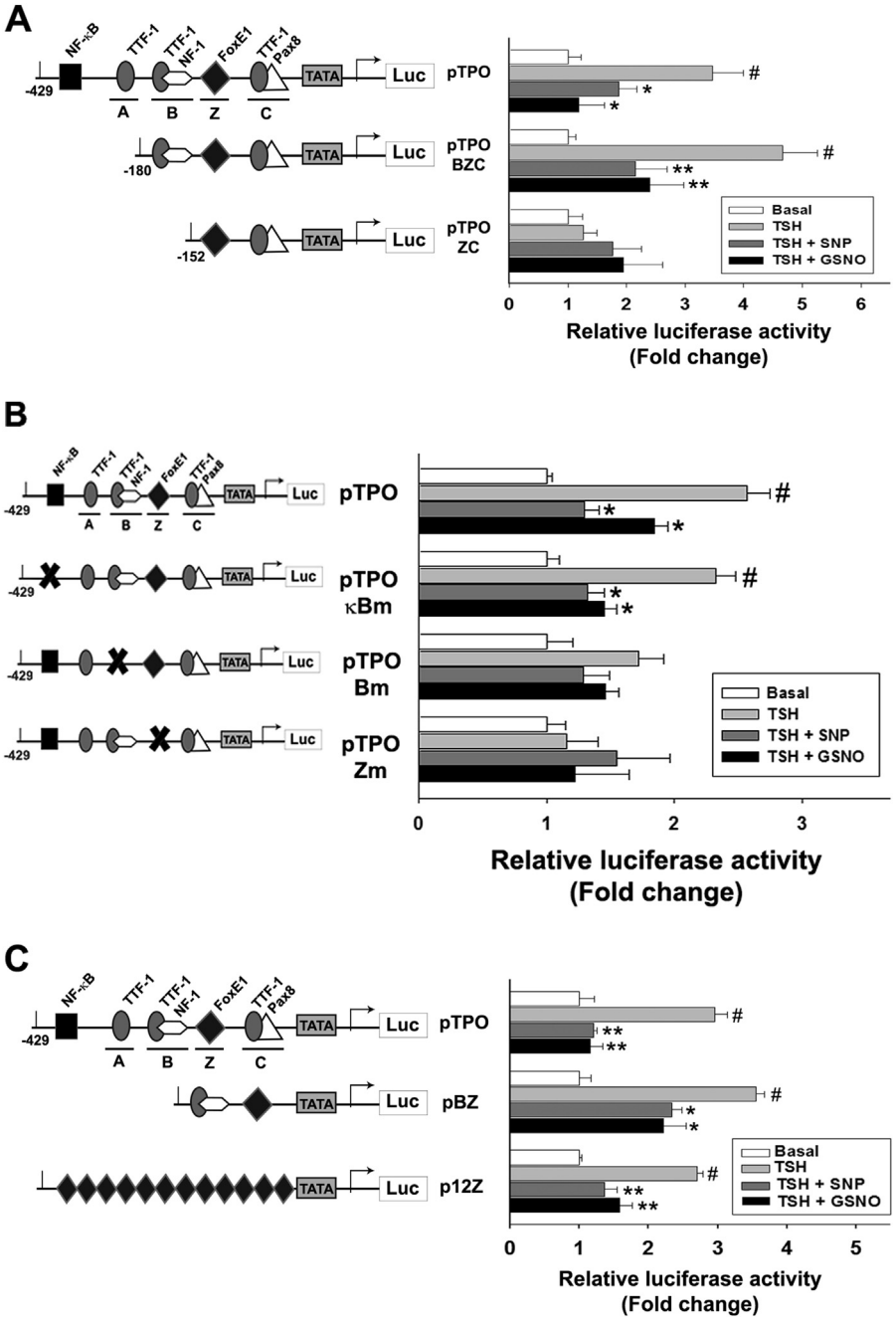


**Fig. 2. NO donors downregulate TSH-dependent TPO gene transcriptional activation involving the Z site**. FRTL-5 cells were transiently transfected with different TPO promoter constructs linked to the reporter gene luciferase (Luc). The *left panel* shows transfected constructs, and the *right panel* shows transcriptional activity in response to the indicated treatment. Starved cells were treated with SNP (200 μM) or GSNO (200 μM) in the presence of TSH (0.5 mIU/ml) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. **A**, #$p < 0.001$ vs Basal; *$p < 0.01$, **$p < 0.001$ vs TSH (ANOVA, Student–Newman–Keuls) **B**, #$p < 0.001$ vs Basal; *$p < 0.001$ vs TSH (ANOVA, Student–Newman–Keuls). **C**, #$p < 0.01$ vs Basal; *$p < 0.01$, **$p < 0.001$ vs TSH (ANOVA, Student–Newman–Keuls).

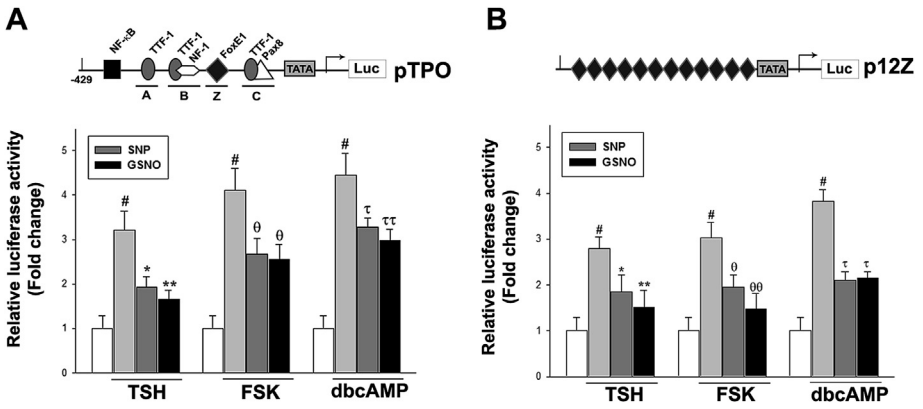


**Fig. 3. NO donors downregulate cAMP-stimulated TPO gene expression**. FRTL-5 cells were transiently transfected with **A**, the TPO promoter construct pTPO or **B**, the FoxE1 reporter containing twelve Z elements in tandem (p12Z). Starved cells were treated with SNP (200 μM) or GSNO (200 μM) in the presence of TSH (0.5 mIU/ml), forskolin (FSK, 10 μM) or dibutyryl-cAMP (dbcAMP, 1 mM) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #p < 0.001 vs Basal; *p < 0.01, **p < 0.001 vs TSH; θp < 0.01; θθp < 0.001 vs FSK; τp < 0.01, ττp < 0.001 vs dbcAMP (ANOVA, Student–Newman–Keuls).

results provide evidence suggesting that NO-repressed TSH-dependent TPO gene expression involves the cis-element Z.

### 3.3. NO donors downregulate cAMP-stimulated TPO gene expression

TSH stimulates TPO expression mainly through the cAMP/PKA signal pathway (Aza-Blanc et al., 1993; Gerard et al., 1988; Postiglione et al., 2002). Therefore, we analyzed the effect of NO donors on FSK- or dbcAMP-stimulated TPO expression. FRTL-5 cells were transfected with the pTPO reporter and then stimulated with 0.5 mIU/ml TSH, 10 μM FSK or 1 mM dbcAMP in the presence or absence of NO donors. As expected, FSK and dbcAMP fully mimicked TSH-induced pTPO promoter activity (Fig. 3A). In addition, SNP and GSNO reduced FSK- and dbcAMP-increased TPO promoter activity (Fig. 3A) thus suggesting that the effect of NO occurs downstream of TSHR activation and cAMP synthesis. Interestingly, similar results were observed in p12Z-transiently transfected cells (Fig. 3B) supporting the involvement of the Z binding site in the effect of NO donors. Exposure of FRTL-5 cells to SNP and GSNO did not modify TSHR levels, disregarding changes in TSHR expression by NO donors (data not shown).

### 3.4. NO-repressed TSH-stimulated TPO expression involves the cGMP/cGK pathway

To assess the specific role of NO after NO donors addition, we tested the effect of the widely used stable radical scavenger for NO PTIO (Maeda et al., 1994). Thyroid cells were transiently transfected with the TPO promoter vector pTPO or the FoxE1 reporter p12Z, starved and further stimulated with TSH alone or TSH plus PTIO in the presence or absence of NO donors. As shown in Fig. 4A, PTIO blocked the inhibitory effect of NO donors on TSH-stimulated pTPO and p12Z activity, thus confirming the specific role for NO in the regulation of TPO gene expression. Nor was any significant effect of PTIO observed on TSH-stimulated pTPO or p12Z transcriptional activity (Fig. 4A).

To study whether sGC and PKG are involved in the NO-repressed TSH-stimulated TPO expression, we used the specific sGC inhibitor ODQ and the cGK inhibitors KT5823 and Rp. Cells transiently transfected with the TPO promoter vector pTPO were stimulated with TSH alone or TSH plus 20 μM ODQ, 10 μM KT5823 or 100 μM Rp in the presence of NO donors for 24 h. We observed that the inhibitors of both enzymes blocked the effect exerted by NO donors on the TSH-stimulated pTPO activity (Fig. 4B), indicating the involvement of the cGS/cGK pathway in the inhibitory effect of NO. Moreover, ODQ, KT5823 and Rp treatment did not significantly modulate TSH-stimulated pTPO transcriptional activity (data not shown).

### 3.5. NO donors inhibit TSH-induced FoxE1 binding to TPO promoter

Our experimental data suggest that the FoxE1 binding site Z is involved in the NO donors-repressed TPO promoter activity. Therefore, we investigated NO donors-induced changes in FoxE1 binding to the TPO promoter using EMSA assays. Nuclear extracts from thyroid cells treated with TSH in the presence or absence of SNP were incubated with $^{32}$P-labeled TPO-Z oligonucleotide. As shown in Fig. 5A, TSH increased the two upper DNA-protein complexes (lanes 1 and 2). Binding specificity was assessed by complete displacement of the shifted band with an excess of the unlabeled TPO-Z oligonucleotide (Fig. 5A, lane 3). Moreover, $^{32}$P-labeled Z-mutated oligonucleotide did not form complexes when incubated with FRTL-5 nuclear extracts (Fig. 5A, lane 6). We observed a significant decrease in the TSH-induced DNA-protein complex in SNP-treated cells (lane 5). Supershift assays using a specific anti-FoxE1 antibody demonstrated a reduction in the shifted band suggesting the participation of FoxE1 in the protein complex with the probe TPO-Z (Fig. 5A, lanes 4 and 7).

We further assessed the interaction between FoxE1 and the TPO promoter using ChIP assays. FRTL-5 cells were stimulated with TSH in presence or absence of NO donors for 1 h. After cross-linking and sonication, soluble chromatin was immunoprecipitated using a monoclonal antibody against FoxE1. Immunoprecipitated DNA was analyzed by qPCR with specific primers designed to amplify the region BZ in the TPO promoter (Fig. 5B). In line with previous reports (Cuesta et al., 2007; Fernandez et al., 2013), quantitative ChIP analysis revealed a strong interaction of FoxE1 with the TPO promoter in the presence of TSH (Fig. 5C). Conversely, NO donors' treatment significantly reduced the TSH-induced binding of FoxE1 to the TPO promoter. Together, our findings indicate that NO donors decrease the TSH-stimulated binding of FoxE1 to the Z region within the TPO promoter.

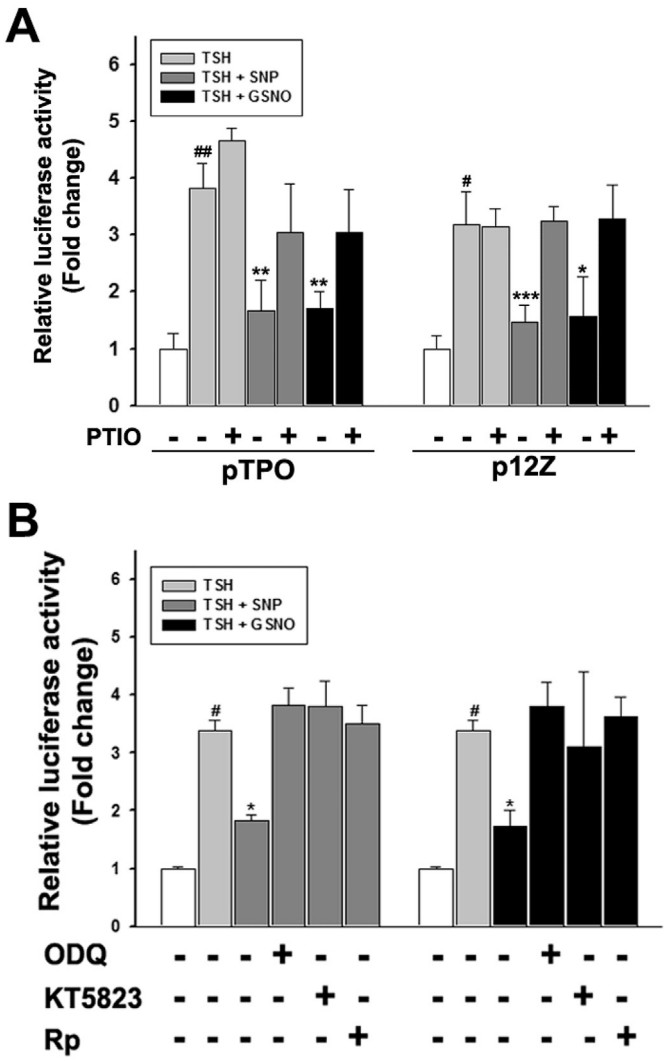


**Fig. 4. NO-repressed TSH-stimulated TPO expression involves cGMP/cGK pathway.** **A-** FRTL-5 cells were transiently transfected with the TPO promoter construct pTPO or the FoxE1 reporter p12Z. Starved cells were treated with SNP (200 μM) or GSNO (200 μM) in the presence of TSH (0.5 mIU/ml) plus the NO scavenger PTIO (200 μM) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity for each construct. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #p < 0.01, ##p < 0.001 vs Basal; *p < 0.05; **p < 0.01; ***p < 0.001 vs TSH (ANOVA, Student–Newman–Keuls). **B-** FRTL-5 cells were transiently transfected with the TPO promoter construct pTPO, TSH-starved, and further treated with SNP (200 μM) or GSNO (200 μM) in the presence of TSH (0.5 mIU/ml) plus the specific sGC inhibitor ODQ (20 μM) or the cGK inhibitors KT5823 (10 μM) and Rp (100 μM) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #p < 0.001 vs Basal; *p < 0.01 vs TSH (ANOVA, Student–Newman–Keuls).

### 3.6. NO donors repress TSH-induced FoxE1 expression

Cuesta et al. (2007) reported that FoxE1 expression closely correlates with its binding to the TPO promoter. Therefore, we evaluated modifications in FoxE1 expression in response to NO donors. As shown in Fig. 6A, TSH stimulation induced a significant up-regulation of FoxE1 protein expression. In contrast, treatment of thyroid cells with different concentrations of either SNP or GSNO decreased TSH-induced FoxE1 protein expression. Consistently, the NO donor SNP repressed the TSH-stimulated FoxE1 mRNA expression in a dose-dependent manner (Fig. 6B). Similar results were observed under GSNO treatment (Fig. 6C). Treatment with SNP or GSNO alone did not modify the basal levels of FoxE1 protein and mRNA expression (data not shown).

To study the molecular mechanism by which NO donors inhibited TSH-induced FoxE1 expression, we analyzed the transcriptional activity of the FoxE1 promoter. Thyroid cells were transiently transfected with a construct containing a 2489 bp fragment of the rat FoxE1 promoter linked to the reporter Luc, starved, and further stimulated with TSH in the presence or absence of NO donors for 24 h. As expected, we observed that TSH induced a significant increase of FoxE1 promoter activity (Fig. 6D and E). Consistently, we observed that SNP and GSNO inhibited the TSH-stimulated FoxE1 promoter activity in a dose-dependent manner (Fig. 6D and E). The functional inhibition of the FoxE1 promoter by NO donors was in line with the inhibitory effects observed on FoxE1 protein and mRNA levels. All together, these results indicate that increased NO levels inhibited TSH-dependent FoxE1 expression involving a transcriptional repression, thus reducing FoxE1 binding to TPO promoter and TPO gene expression.

### 3.7. FoxE1 overexpression restores NO donors-repressed TPO promoter activity

To further test our hypothesis, FRTL-5 cells were cotransfected with the TPO promoter construct pTPO along with either an empty vector or an expression vector encoding FoxE1 under the control of a constitutive promoter. After starvation, transfected cells were stimulated with TSH in the presence or absence of NO donors for 24 h. Interestingly, FoxE1 overexpression prevented the inhibition of NO donors on TSH-stimulated TPO promoter activity (Fig. 7). It is worth noting that FoxE1-overexpressing thyroid cells require TSH stimulation to up-regulate TPO gene expression. All together, these data indicate that NO-mediated transcriptional repression of FoxE1 constitutes an essential step to regulate TPO transcriptional expression.

## 4. Discussion

Thyroid hormone biosynthesis is a complex multi-step process that takes place within the thyroid follicular cells and the extracellular follicular lumen. TPO catalyzes thyroid hormone synthesis at the apical membrane–colloid interface of thyrocytes by mediating the covalent incorporation of iodine into tyrosine residues of thyroglobulin, process known as iodine organification, and subsequent coupling of iodotyrosyl residues to form thyroid hormones (Ris-Stalpers and Bikker, 2010). The pituitary hormone TSH is the main regulator of thyroid function, although other factors including intracellular iodine levels and follicular thyroglobulin function as autocrine negative regulators of thyroid hormone biosynthesis (Serrano-Nascimento et al., 2014; Suzuki et al., 1999). In addition, endogenously TSH-induced NO synthesis has been implicated in the regulation of the thyroid function (Fozzatti et al., 2007). Kasai et al. (1995) and Costamagna et al. (1998) described a regulatory role of NO in the thyroid hormone biosynthesis by inhibiting iodine organification in primary cultures of human and bovine thyroid follicles. Consistently, NOS II-produced NO partially mediates pro-inflammatory cytokines-repressed TPO expression in human thyrocytes (Gerard et al., 2006; van den Hove et al., 2002). More recently, Bazzara et al. (2007) reported that NO donors inhibit TSH-induced TPO mRNA expression in rat thyroid cells. Here, we provide novel evidence indicating that NO released from NO donors decreases TSH-stimulated TPO gene expression. Moreover, we demonstrated that NO inhibits the expression of the transcription factor FoxE1 in response to TSH, thus leading to TPO transcriptional repression.

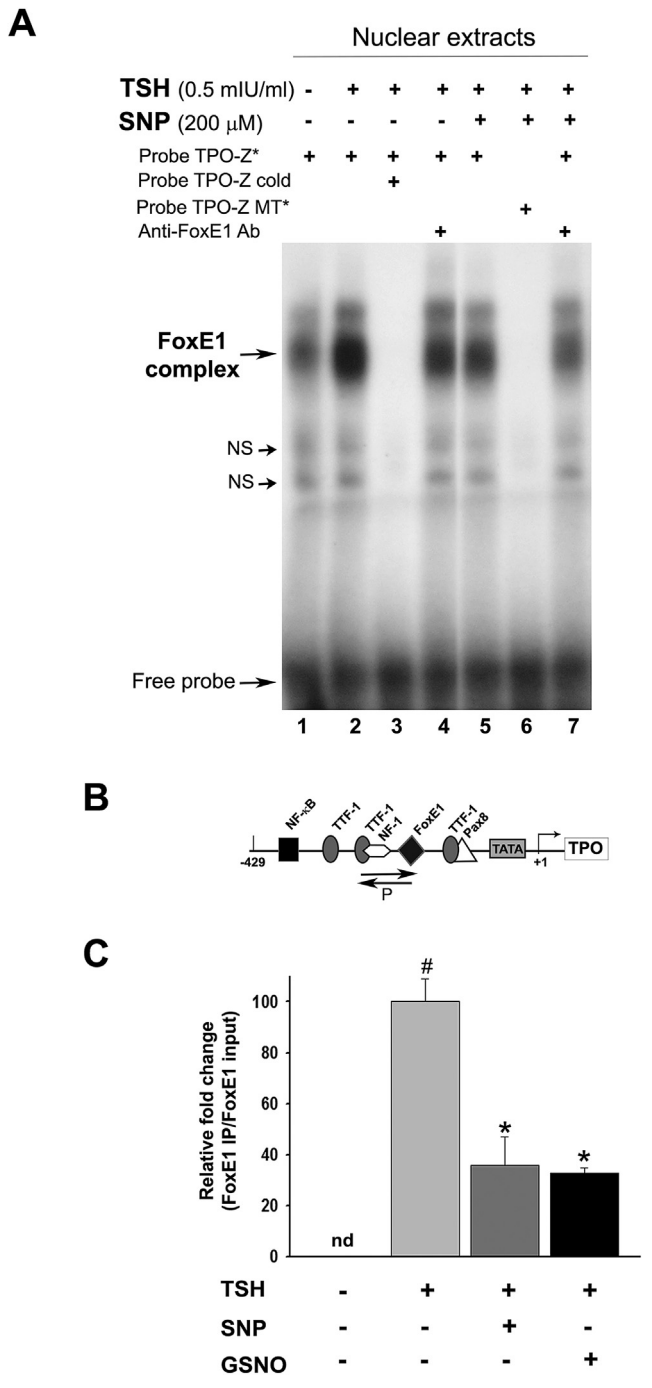


**Fig. 5. NO donors inhibit TSH-induced FoxE1 binding to the TPO promoter. A**, Representative EMSA performed with nuclear extracts obtained from FRTL-5 cells treated with SNP (200 μM) in the presence of TSH (0.5 mIU/ml) for 24 h. EMSAs were performed with $^{32}$P-labeled oligonucleotides corresponding to the FoxE1 binding site Z (Probe TPO-Z*). Specificity was achieved by performing competition reactions in the presence of 100-fold excess of cold TPO-Z oligonucleotide (lane 3), and by the inhibition of shifted complex when the Z binding site is mutated (Probe TPO-Z MT*; lane 6). Incubations performed in the presence of an anti-FoxE1 antibody diminished the formation of specific FoxE1 complex (lanes 4, 7), thus denoting that FoxE1 specifically recognizes the Z site in the TPO promoter region. **B**, Schematic representation of the rat TPO promoter. Positions are relative to the first nucleotide where transcription starts, denoted as +1. The location of the primer set (P) used in ChIP assays is shown. **C**, Starved cells were treated with 200 μM NO donor in the presence of 0.5 mIU/ml TSH for 1 h before cross-linking and a further ChIP assay. Results are expressed as relative fold increase (FoxE1IP/FoxE1input). nd: not detectable. #$p < 0.05$ *vs* basal condition; *$p < 0.05$ *vs* TSH-treated cells.

Thyroid microvasculature plays an important role in controlling thyroid homeostasis. The morphological and functional unit of the thyroid is not restricted to the follicular cells, but also includes endothelial cells from adjacent capillaries. Autocrine/paracrine interactions between follicular and endothelial cells modulate thyroid economy. Throughout goiter formation, not only TSH, but also many locally produced growth, angiogenic and vasoactive factors are involved in the proliferation of epithelial and endothelial cells (Colin et al., 2013). In this regard, NO signaling has been implicated in the regulation of thyroid microvasculature in response to goitrogenic agents (Colin et al., 1995). NOS III is expressed in the thyroid follicular and endothelial cells of the human thyroid gland (Colin et al., 1997), in agreement with a role for NO in the vascular control of the thyroid physiology. Very recently, Craps et al. (2015) demonstrated that iodide deficiency-induced thyroidal blood flow relies on increased NOS III-mediated NO production. In addition, human thyrocytes produce the endogenous NOS inhibitor asymmetric dimethylarginine (Millatt et al., 2000) suggesting a potential control of NOS activity in the thyroid cell.

The present study demonstrated that the NO released from commonly used NO donors inhibited the mRNA and protein expression of TPO in response to TSH in FRTL-5 thyroid cells. NO donors repressed the TSH-dependent TPO promoter activity, suggesting that NO inhibited TPO expression at transcriptional level. We evaluated several serial 5′-deletions of the TPO promoter as well as different site-directed mutants. Deletion of the region −429 to −180 (pTPO BZC) did not affect NO donors-repressed TPO expression, thus suggesting that factors other than NF-κB and TTF-1 may be involved in the NO-triggered effect. TPO promoter cis-regulatory regions B and Z, which comprise binding sites for TTF-1 and FoxE1 respectively, are determinants for full TSH-induced TPO expression (Aza-Blanc et al., 1993; Ortiz et al., 1999). We demonstrated the involvement of FoxE1 binding site Z in the NO-downregulated TPO expression as NO donors repressed the activity of the artificial TSH-responsive construct containing cis-acting sites B and Z (pBZ) and the transcriptional activity of the TSH-responsive FoxE1 reporter containing a tandem repeat of 12 cis-elements Z. Moreover, we used different approaches to characterize the functional role of FoxE1 as mediator of the NO-inhibited TPO expression. EMSA studies demonstrated that the NO donor SNP reduced FoxE1 binding to an oligonucleotide sequence containing the Z region from the TPO promoter. We corroborated the NO-reduced FoxE1 binding to the TPO promoter *in vivo* using quantitative ChIP analysis. These results indicate that the NO-repressed binding of FoxE1 to the TPO promoter could be involved in the inhibited transactivation of the TPO gene in response to NO donors, thus leading to a reduced TPO expression. However, other mechanisms that prevent either FoxE1 binding to the cis-acting element Z or FoxE1-dependent recruitment of transcriptional coactivators could be involved, although this hypothesis awaits confirmation.

In thyroid cells, TPO expression is dependent on the activation of the TSHR/cAMP cascade, as stimulators of the adenylate cyclase/cAMP signaling pathway mimic TSH-induced TPO expression (Gerard et al., 1988, 1989). Pro-inflammatory cytokines-decreased TPO expression (Gerard et al., 2006; Poncin et al., 2008) can be overshadowed by increased TSH concentrations suggesting a mutual antagonism between pro-inflammatory cytokines and TSH-activated intracellular pathways (Gerard et al., 2006). Herein, we studied the effect of NO donors in the presence of adenylate cyclase/cAMP signaling

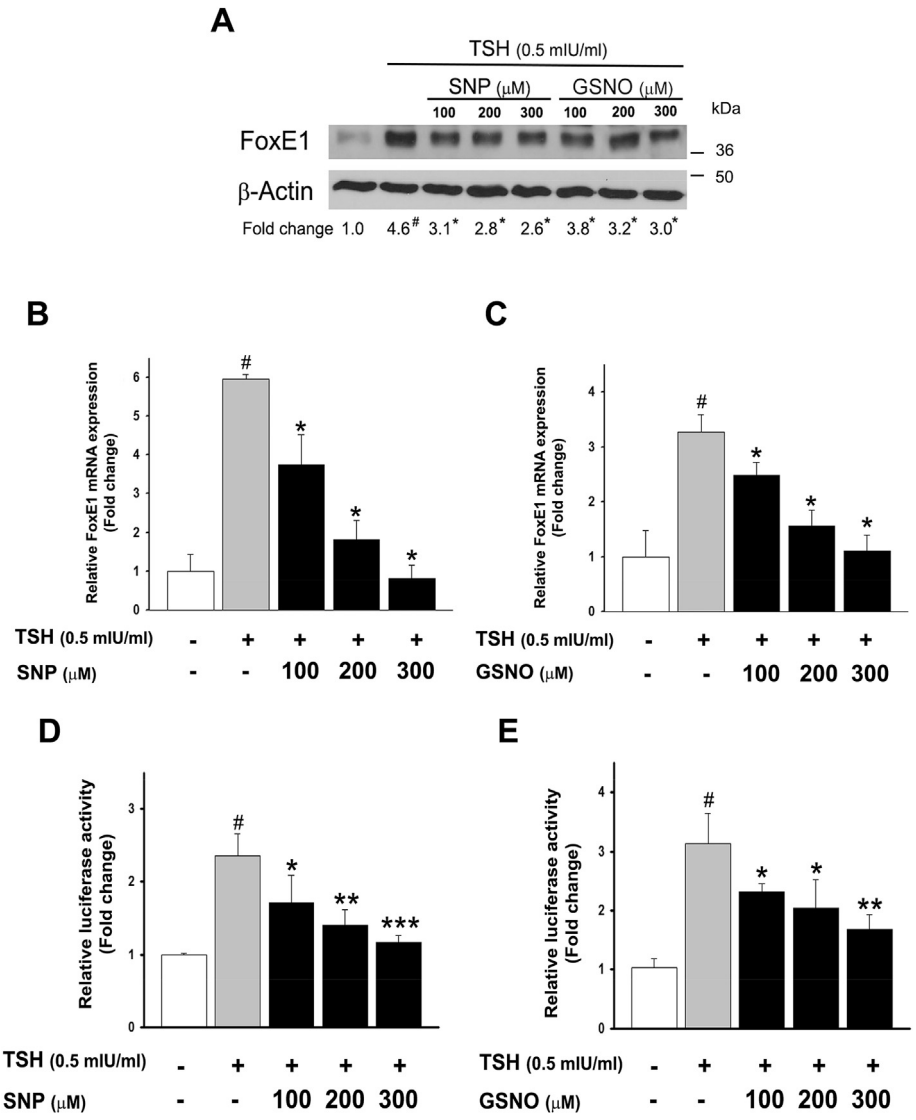


**Fig. 6. NO donors inhibit TSH-induced FoxE1 expression**. Starved FRTL-5 cells were treated with SNP (100–300 μM) or GSNO (100–300 μM) in the presence of TSH (0.5 mIU/ml) for 24 h. **A**, Representative Western blot of whole protein extracts assessing FoxE1 expression. β-Actin expression was used as control for equal protein loading (*lower panel*). Densitometric analysis was performed to determine the relative expression of FoxE1 normalized to β-Actin. Fold change indicates the mean of at least three independent experiments. #$p < 0.01$ vs Basal; *$p < 0.05$ vs TSH (ANOVA; Student–Newman–Keuls). **B** and **C**, Relative FoxE1 mRNA quantification evaluated by RT/qPCR. FoxE1 mRNA expression levels were quantified relative to those of β-Actin. Results are indicated as fold change relative to the mRNA levels of untreated cells. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. **B**, #$p < 0.001$ vs Basal; *$p < 0.01$ vs TSH. **C**, #$p < 0.001$ vs Basal; *$p < 0.05$, **$p < 0.001$ vs TSH (ANOVA; Student–Newman–Keuls). **D** and **E**, FRTL-5 cells were transiently transfected with the FoxE1 promoter construct linked to the reporter gene luciferase (Luc). Starved cells were treated with SNP (100–300 μM) or GSNO (100–300 μM) in the presence of TSH (0.5 mIU/ml) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #$p < 0.001$ vs Basal; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ vs TSH (ANOVA, Student–Newman–Keuls).

activators. We observed that NO donors repressed TPO expression downstream to TSH-stimulated cAMP production. Concordantly, several reports suggested no changes in TSH-triggered cAMP production in response to NO donors (Bazzara et al., 2007; Decoster and Dumont, 1985; Millatt et al., 1993), indicating a possible cross-talk between TSH and NO/cGMP pathways downstream to cAMP production.

Most of the physiological NO effects are mediated by selective activation of sGC, the generation of the secondary messenger cGMP, and the consequent activation of cGKs (Martinez-Ruiz et al., 2011). Several authors reported increased cGMP production in response to NO donors in different thyroid cell models (Bazzara et al., 2007; Bocanera et al., 1997; Esteves et al., 1992; Millatt et al., 1993). Bazzara et al. (2007) reported that cell membrane-permeable cGMP analogs mimics NO effects repressing TSH-stimulated TPO mRNA expression. Accordingly, we demonstrated that either sGC inhibition using the selective chemical inhibitor ODQ or cGK chemical inhibition using the structurally unrelated inhibitors KT5823 and Rp abrogated NO donors-repressed TSH-induced TPO gene expression, thus suggesting the activation of sGC/cGK signaling in response to increased NO levels. Unlike, very recently, we provided evidence supporting that NO-modulated $Na^+/I^-$ symporter (NIS)-mediated iodide accumulation in thyroid cells involves S-nitrosylation of the NF-κB subunit p65, which reduces trans-activation of NIS gene expression in response to TSH stimulation (Nicola et al., 2015).

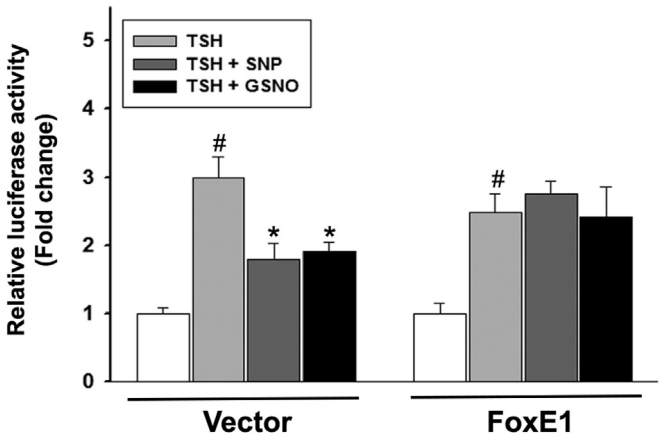


**Fig. 7. FoxE1 overexpression restores NO donors-repressed TPO promoter activity.** FRTL-5 cells were transiently cotransfected with the TPO promoter pTPO together with an empty vector (Vector) or a FoxE1 expression vector (FoxE1). Transfected cell were starved and treated with SNP (200 μM) or GSNO (200 μM) in the presence of TSH (0.5 mIU/ml) for 24 h. Results are expressed as Luc activity normalized to β-galactosidase and relative to basal activity. Data (mean ± SD) are from a representative experiment done in triplicate from a total of three with similar results. #$p < 0.001$ vs Basal; *$p < 0.01$ vs TSH (ANOVA, Student–Newman–Keuls).

FoxE1 is a thyroid-specific transcription factor that belongs to the Fox family of transcription factors (Zannini et al., 1997). FoxE1 is essential during thyroid morphogenesis and differentiation in the fetus, as well as for the maintenance of the thyroid differentiated state in adults (Fernandez et al., 2015). The importance of FoxE1 in the maintenance of the thyroid differentiated state is related to its role in controlling the expression of several thyroid hormone biosynthesis-related genes (Fernandez et al., 2013). Fox proteins are considered pioneer transcription factors as being able to bind condensed chromatin structure, creating locally exposed domains necessary for the action of other transcription factors. Indeed, Cuesta et al. (2007) demonstrated that FoxE1 binding to the cis-acting element Z within the TPO promoter creates a local open binding domain enabling other factors to access the chromatin to initiate TPO gene transcription. FoxE1 synergistically interacts with the constitutive transcription factor CTF/NF1 to promote TPO gene expression in response to TSH stimuli (Ortiz et al., 1999). FoxE1 binding site Z within the TPO promoter seems to become occupied after TSH induction only when FoxE1 expression is evident (Cuesta et al., 2007). Our observations indicated that NO is able to reduce TSH-stimulated FoxE1 protein and mRNA expression. Moreover, evaluating FoxE1 promoter activity, we obtained evidence indicating that NO-downregulated FoxE1 expression takes place at transcriptional level. These findings support the inhibitory effect of NO on the TPO gene transcriptional activity might result as a consequence of a decreased FoxE1 level. Similarly, Miyazaki et al. (1999) suggested that pro-inflammatory cytokines-induced suppression of TPO expression involves a reduced gene expression and DNA-binding of FoxE1. It is known that TSH increases FoxE1 mRNA expression through the cAMP pathway requiring ongoing protein synthesis in FRTL-5 cells, although the molecular mechanism underlying this effect is still poorly understood (Zannini et al., 1997). Further studies are necessary to gain insights into the mechanism of FoxE1 regulation.

The NO-mediated cellular effects are dependent on its concentration and duration of exposure as well as the expression profile, activity and localization of NOS isoforms and cellular sensitivity to NO. NOS II can be induced by a variety of inflammatory cytokines and can produce micromolar levels of NO. However, NOS III which generates low levels of NO for very short-time periods, also plays a role in inflammation (Burke et al., 2013). Previously, we provided evidence indicating that TSH-stimulated endogenously produced NO might act as an autocrine counter-regulatory mechanism to restrain TSH-stimulated thyroid cell function and proliferation (Fozzatti et al., 2007). Consistently, Colin et al. (1997) reported increased NOS III protein expression in thyroid tissues derived from patients with Graves' disease and autonomous toxic adenomas carrying activating mutations in the TSHR. Hence, it appears that both the thyrocyte-produced low level of NO and the NO donor–released high level of NO are able to exert an inhibitory action on thyroid cell activity.

In conclusion, we provide evidence revealing a NO-triggered inhibitory effect of TSH-stimulated TPO gene expression. The NO-regulated TPO expression could explain the observed changes in thyroidal iodide organification in response to increased intracellular NO level. Mechanistically, we demonstrated that NO decreases TSH-induced FoxE1 gene expression, thus repressing the transcripcional activation of TPO gene. Importantly, NO-regulated FoxE1 expression may be relevant in modulating FoxE1-dependent transcriptional networks in differentiated thyroid cells (Fernandez et al., 2013). In addition, NO-mediated long term inhibition of thyroid differentiation could be of interest in relation to human thyroid pathologies associated with a chronic NO production. Taken together, the results presented here reinforce the regulatory role of NO on thyroid cell function that could be of pathophysiological relevance.

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## Disclosure statement

The authors have nothing to disclose.

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