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Nitric Oxide/cGMP Signaling Inhibits TSH-Stimulated Iodide Uptake and Expression of Thyroid Peroxidase and Thyroglobulin mRNA in FRTL-5 Thyroid Cells

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Objective: Nitric oxide (NO) induces morphological and functional alterations in primary cultured thyroid cells. The aim of this paper was to analyze the direct influence of a long-term exposition to NO on parameters of thyroid hormone biosynthesis in FRTL-5 cells. *Design*: Cells were treated with the NO donor sodium nitroprusside (SNP) for 24–72 h. *Main Outcome*: SNP (50–500 μmol/L) reduced iodide uptake in a concentration-dependent manner. The inhibition of iodide uptake increased progressively with time and matched nitrite accumulation. SNP inhibited thyroperoxidase (TPO) and thyroglobulin (TG) mRNA expression in a concentration-dependent manner. SNP enhanced 3′,5′-cyclic guanosine monophosphate (cGMP) production. 3′,5′-cyclic adenosine phosphate (cAMP) generation was reduced by a high SNP concentration after 48 h. 8-Bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), a cGMP analog, inhibited iodide uptake as well as TPO and TG mRNA expression. The cGMP-dependent protein kinase (cGK) inhibitor KT-5823 reversed SNP or 8-Br-cGMP-inhibited iodide uptake. Thyroid-stimulating hormone pretreatment for 24–48 h prevented SNP-reduced iodide uptake although nitrite levels remained unaffected. *Conclusion*: These findings favor a long-term inhibitory role of the NO/cGMP pathway on parameters of thyroid hormone biosynthesis. A novel property of NO to inhibit TPO and TG mRNA expression is supported. The NO action on iodide uptake could involve cGK mediation. The long-term inhibition of steps of thyroid hormonogenesis by NO could be of interest in thyroid pathophysiology.

Introduction

Nitric oxide (NO), a free radical reactive molecule, plays an important role in signal transduction. Over the last years, increasing reports have involved NO in many important physiologic and pathologic processes, including blood pressure regulation, inflammatory reaction, and neurotransmission (1–4). NO is generated endogenously from L-arginine by NO synthases (NOS), a family with at least three different isoforms expressed in numerous tissues. The neuronal (nNOS, NOS I) and the endothelial (eNOS, NOS III) isoforms are constitutive and Ca^{2+}-dependent enzymes, whereas the macrophage or inducible NOS (iNOS, NOS II) is Ca^{2+}-independent and regulated at transcriptional level by numerous agents (3–7). It is generally accepted that the constitutive isoforms NOS I and III generate low levels of NO which are associated with homeostatic and regulatory functions, whereas after NOS II induction, a high and sustained NO production is obtained which is involved in cytotoxic and cytostatic actions (2,3,7). The activation of the soluble guanylyl cyclase that results in the formation of 3′,5′-cyclic guanosine monophosphate (cGMP) from guanosine triphosphate has received primary consideration as the main mechanism of NO actions. Guanylyl cyclase exists in two major forms, the soluble enzyme that has been assumed to be the receptor for NO and the particulate guanylyl cyclases that are the membrane-bound natriuretic peptide receptors (3,4,8). Several different downstream mechanisms for the cGMP-mediated regulation of cellular responses have been proposed such as protein phosphorylation by the cGMP-dependent protein kinase (cGK) (3,4). A role of cGMP in the regulation of gene expression has been recognized, and a growing list of cGMP-regulated genes is reported (3,9–11).

Previous studies have addressed relationships between NO and the thyroid gland. Increased cGMP accumulation after Ca^{2+} cascade stimulation in dog thyroid slices has been observed (12), and it was later demonstrated to involve the activation of the soluble guanylyl cyclase by the NOS-

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mediated production of NO (13). The three NOS isoforms were identified in the rat thyroid tissue (14). Changes of NOS expression have been demonstrated in several pathophysiological conditions of the thyroid gland (14–16). A modulation of NOS II expression (17,18) and NO production (17–19) by cytokines has been observed in human thyrocytes.

A reduction of iodide transport and organification after 2–4 h of treatment with the NO donor sodium nitroprusside (SNP) in human (17) and bovine (20) thyroid primary cultured cells has been reported. We have previously demonstrated that the long-term treatment with the NO donors SNP and S-nitrosoglutathione inhibited iodide uptake, organification, and transport, as well as induced morphological changes in bovine thyroid cells (21). The production of cGMP was increased by NO donors in thyrocytes of several species (13, 20–23). The mediation of the guanylyl cyclase/cGMP pathway in the action of NO on thyroid cells has been proposed (20,24).

The aim of this work was to investigate the effect of the long-term exposition to NO on iodide uptake and the expression of the thyroid-specific genes thyroperoxidase (TPO) and thyroglobulin (TG) in the rat thyroid cell line FRTL-5. These cells retain most of the features of differentiated follicular thyroid cells (25,26) and are deprived of other cell types being particularly useful to examine the direct action of NO on thyrocytes. Here we demonstrate for the first time that the NO supplied exogenously to thyrocytes is able to inhibit the thyroid-stimulating hormone (TSH)–induced TPO and TG gene mRNA expression. A reduction of TSH-stimulated iodide uptake by NO was observed, and it seems to be mediated by cGK. It was found that NO increased cGMP levels, whereas cAMP level was reduced at a high NO concentration after a long period of incubation. The effects of NO were mimicked by an analog of cGMP, supporting the involvement of the guanylyl cyclase/cGMP pathway in the action of NO. The pretreatment of cells with TSH blocked the inhibitory effect of SNP on iodide uptake, indicating a less-efficient action of NO when the TSH-induced signaling pathways have been previously stimulated.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham F-12 medium and calf serum were provided by GIBCO BRL (Gaithersburg, MD) and tissue culture dishes by Corning Glass Works (New York) or Falcon (Devon, UK). ^{131}Iodine sodium iodide ($Na^{131}I$) was obtained from Comisión Nacional de Energía Atómica (Buenos Aires, Argentina), and [α-32P]dATP was provided by NEN Life Science Products (Boston, MA). Random primers labeling kit was obtained from Amersham Pharmacia Biotech (Buenos Aires, Argentina). Bovine TSH (thyrotropin) was generously provided by the National Hormone and Pituitary Program, NIDDK, and Dr. Parlow, NIH (Baltimore, MD). Transferrin, insulin, glutamine, N2, 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), isobutylmethylxantine (IBMX), guanidinium isothiocyanate, and herring testes DNA were from Sigma Chemical (St. Louis, MO). KT-5823 was obtained from Calbiochem (La Jolla, CA). cAMP and cGMP RIA kits were from Immunotech (Marseille, France). All chemicals and reagents were of analytical grade.

Culture of FRTL-5 cells

The growth and maintenance of the continuous rat thyroid cell strain FRTL-5 (ATCC CRL 8305; American Type Culture Collection, Manassas, VA), kindly provided by Dr. L. Kohn (NIH, Bethesda, MD), has been previously described (27). The FRTL-5 thyroid cells were grown in a 5% carbon dioxide (CO_2)–95% air atmosphere in DMEM/Ham F-12 (1:1) medium supplemented with calf serum (5% v/v), bovine insulin (10 μg/mL), bovine transferrin (5 μg/mL), glutamine (2 mmol/L), bovine TSH (1 mU/mL), antibiotics, and antimycotics. The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. They were passaged every 8–10 days and fed a fresh medium every 2–3 days. When the cells reached 70–80% confluence, the medium was replaced for medium devoid of TSH for 5–7 days before the experiments. Cells deprived of TSH were treated with the indicated concentrations of SNP in the presence of 0.5 mU/mL TSH for different periods of time described (24).

Nitrite assay

To estimate the NO generation induced by the NO donor, the production of nitrite, a stable oxidative metabolite of NO, was measured in the culture medium (21). For the assay, 100 μL of culture medium was reacted with 200 μL Griess reagent (1.5% sulfanilamide in HCl 1N:5.5 mmol/L α-naphthylenediamine, 1:1) and incubated for 10 min at room temperature (21). Each experimental point was performed in quadruplicate. The absorbance at 550 nm was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in the culture medium. The results were normalized by μg DNA and expressed as nmol/μg DNA.

Iodide uptake

The ^{131}I uptake assay was performed as previously described (24). FRTL-5 cells were incubated with 500 μL Hank's Buffered Salt Solution (HBSS) containing 0.1 μCi carrier-free Na131I and 1 μmol/L potassium iodide for 35 min at 37°C, washed twice with ice-cold HBSS, gently scraped, and centrifuged at 1500 g for 10 min. Iodide uptake was determined by measuring the total radioactivity incorporated to the cells and expressed as counts per min (cpm)/μg DNA.

RNA isolation, Northern blot analysis, and RT-PCR assay

Total RNA was purified by the acid guanidinium method described by Chomczynski and Sacchi (28). Cultured thyroid cells were harvested in a denaturing solution containing 4 mol/L guanidine thiocyanate. The suspension was mixed sequentially with 2 mol/L sodium acetate (pH 4), phenol, and chloroform/isoamyl alcohol. The mixture was centrifuged, yielding an upper aqueous phase containing total RNA. Following isopropanol precipitation, the RNA pellet was redissolved in the denaturing solution, reprecipitated with isopropanol, washed with 75% ethanol, and dissolved in RNase-free water. For Northern blot analysis, total RNA (20 μg/lane) was electrophoresed on 1% agarose gel containing 0.66 mol/L formaldehyde. RNA was transferred to nylon membranes by capillary transblotting overnight and then baked for 2 h at 80°C, followed by a prehybridization at 42°C in a

solution containing 50% formamide, 5× Denhardt's solution, 5× SSPE, (43.5 g NaCl/6.9 g $NaH_2PO_4 \cdot H_2O$/1.85 g EDTA per L, pH 7.4) 1% sodium dodecyl sulfate (SDS), and 200 μg/mL heat-denatured herring testes DNA. Hybridizations were performed overnight at 42°C in the same solution containing a ^{32}P-labeled rat TPO (29) or bovine TG (30) cDNA probes. Blots were then washed in 2× SSC (17.53 g NaCl/8.82 g sodium citrate per L, pH 7.0)/1% SDS at room temperature, followed by two washes in 2× SSC/1% SDS and 0.2× SSC/1% SDS at 55°C and exposed to Kodak X-Omat film for 3 days (TPO), 5 days (TG), or 6 h (ribosomal RNA, rRNA) at −70°C. To normalize for possible unequal loading of RNA in the gel, the blots hybridized with TPO or TG were stripped and rehybridized with a 18S rRNA recognizing probe. The band intensities were determined by scanning densitometry (Shimadzu Dual-Wavelength Chromato Scanner CS-930) at 500 nm, and the absorbances of the TPO and TG signals were expressed in arbitrary densitometric units normalized to that of the 18S rRNA in the same lane. RT-PCR assays to estimate the constitutive genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were performed as previously described (31).

cGMP and cAMP assays

After the indicated treatments of FRTL-5 cells in the presence of 0.5 mmol/L IBMX, the medium was collected, immediately frozen, and stored at −70°C. The extracellular cyclic nucleotides were measured by radioimmunoassay, according to the manufacturer's instructions, and the results expressed as pmol cAMP or fmol cGMP per μg DNA.

DNA determination

DNA content was measured in the cellular pellet by diphenylamine method of Burton (32) modified by Kohn and Valente (33).

Statistical analysis

The analysis of multiple intergroup differences was conducted by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls or Dunn's tests. Differences were considered significant at $p < 0.05$.

Results

NO production from SNP in cultured FRTL-5 thyroid cells

To evaluate the ability of SNP to be metabolized in our experimental system to release NO, we estimate the NO generation by quantification of nitrite accumulation, the major stable metabolite of NO degradation, in the culture medium of FRTL-5 cells treated with SNP for 24–72 h. We observed that the NO donor (50–500 μmol/L) induced an increase in the nitrite level in the culture medium of TSH-stimulated cells, which varied with the SNP concentration and the period of incubation, displaying a higher accumulation with the progressive increment in the NO donor concentrations and periods of SNP treatment (Table 1). The viability of the cells, evaluated by the trypan blue dye exclusion test, showed no significant changes in SNP (50–500 μmol/L)–treated cells with respect to that of cells incubated with TSH alone at any time point (98–100% of viable cells). Thus, a sustained NO production for long periods of time was reached in the experimental conditions used. The unaffected survival of the cells offered the possibility to study the NO action on thyroid cell function in cells chronically exposed to NO.

Table 1. Nitrite Accumulation after Treatment of FRTL-5 with the NO Donor SNP

	Nitrite (nmol/μg DNA)		
SNP (μmol/L)	*24 h*	*48 h*	*72 h*
0	0.03 ± 0.02	0.02 ± 0.03	0.03 ± 0.03
50	0.60 ± 0.03^{a}	$1.06 \pm 0.06^{a,b}$	$1.16 \pm 0.09^{a,b}$
100	0.69 ± 0.02^{a}	$1.37 \pm 0.06^{a,b}$	$1.35 \pm 0.10^{a,b}$
500	1.31 ± 0.04^{a}	$1.62 \pm 0.08^{a,c}$	$2.31 \pm 0.29^{a,b,d}$

[a] $p < 0.001$ versus TSH alone (without SNP).
[b] $p < 0.001$ and [c] $p < 0.01$ versus SNP 24 h.
[d] $p < 0.01$ versus SNP 48 h.
Nitrite level was determined in culture medium as described in "Materials and Methods" section. Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. NO: nitric oxide; SNP: sodium nitroprusside; TSH: thyroid-stimulating hormone.

SNP inhibits the TSH-stimulated iodide uptake

To study the effect of a long-time exposition to NO on the regulation of thyroid function, we evaluated the iodide uptake, a crucial step in thyroid hormone biosynthesis, in FRTL-5 thyroid cells treated with different SNP concentrations for different periods of incubation. As shown in Figure 1A, the treatment of FRTL-5 cells with SNP (50–500 μmol/L) in the presence of TSH (500 μU/mL) during 24 h inhibited significantly the TSH-stimulated iodide uptake in a concentration-dependent manner, reaching almost basal levels (without TSH) at 500 μmol/L SNP. The magnitude of the inhibition correlated with the nitrite level in the culture medium ($r = 0.759$, $p < 0.001$). In accordance, a significant inhibition of the TSH-induced iodide uptake was observed after treatment of the cells with 100 μmol/L SNP for 24–72 h, depicting in the iodide uptake a progressive reduction with the increase in the period of incubation (Fig. 1B). SNP had no effect on basal (without TSH) iodide uptake at any assayed concentration or time point; to simplify, the action of the maximal SNP concentration for 24 h on basal cells is shown (Fig. 1A).

SNP inhibits the TSH-induced TPO and TG mRNA expression

The effect of chronic exposition of thyroid cells to NO on thyroid-specific gene expression was analyzed. We studied the action of SNP on the expression of two key genes considered markers of thyroid differentiation: the TPO, enzyme responsible for iodide organification, and TG, the matrix protein for thyroid hormone formation. The cells were incubated with SNP (50–500 μmol/L) in the presence of TSH (500 μU/mL) for 24 h, and the levels of TPO and TG mRNAs were evaluated by Northern blot. We observed that the NO donor significantly reduced the TSH-stimulated TPO and TG mRNA expression in a concentration-dependent manner, with a drastic decrease in the levels of both mRNAs at 500 μmol/L SNP

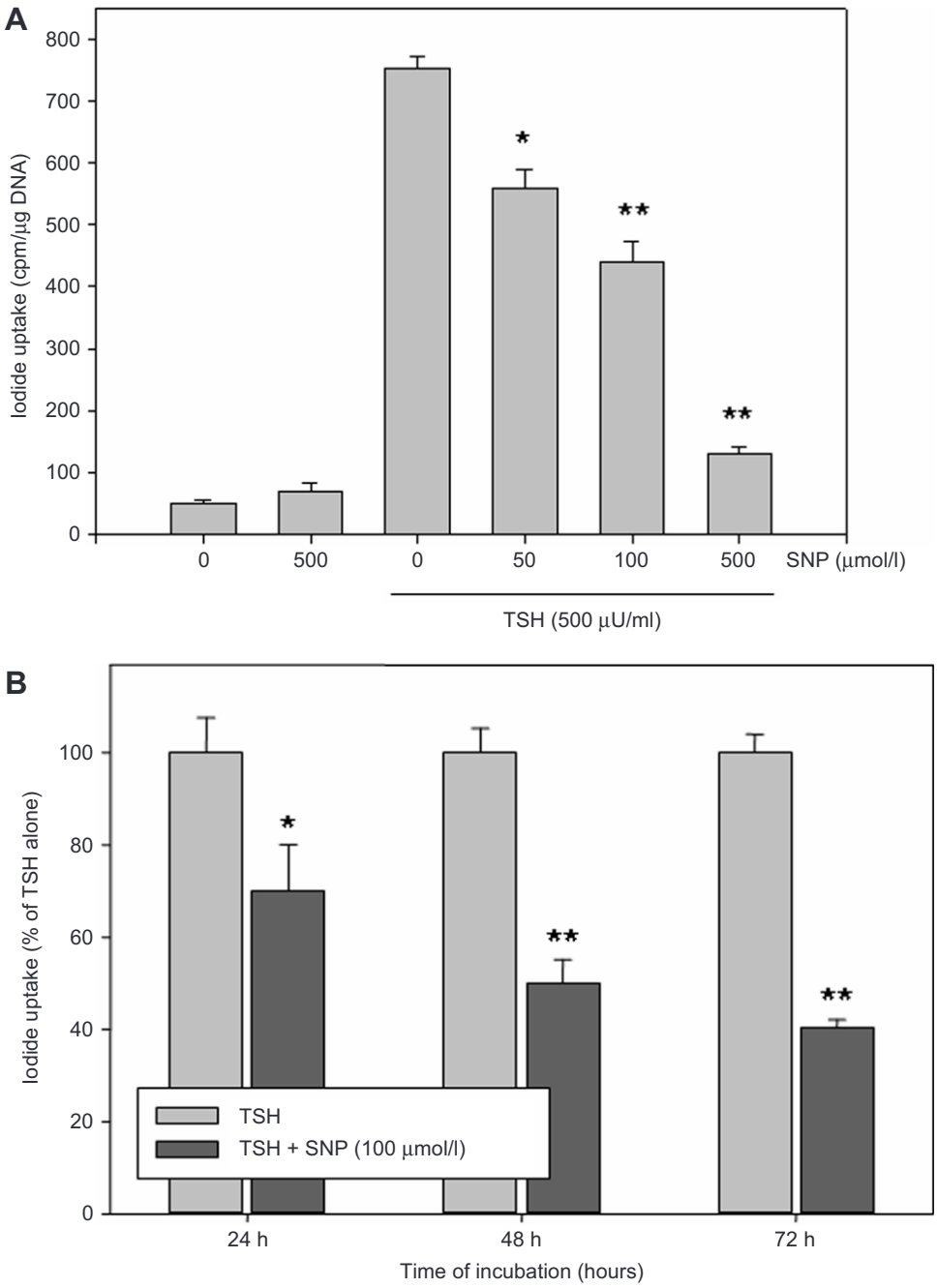

FIG. 1. Sodium nitroprusside (SNP) treatment inhibits the thyroid-stimulating hormone (TSH)–induced iodide uptake. (**A**) Basal FRTL-5 cells were incubated with TSH (500 μU/mL) in the presence or absence of SNP (50–500 μmol/L) for 24 h. Values are expressed as counts per min (cpm)/μg DNA. (**B**) Basal FRTL-5 cells were incubated with TSH (500 μU/mL) in the presence or absence of 100 μmol/L SNP for 24, 48, and 72 h. Values are expressed as percentage of TSH alone value (taken as 100%). Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. *$p < 0.01$ and **$p < 0.001$ versus TSH alone.

(Fig. 2A, B). SNP did not alter basal TPO or TG mRNA expression (data not shown). As NO modified the expression of TPO and TG mRNA in a rather similar way, we tested the specificity of the NO effect by analyzing the expression of the unrelated housekeeping genes β-actin and GAPDH. We observed that NO did not modify β-actin or GAPDH mRNA expression (Fig. 2C, D), supporting that NO does not exert a general unspecific effect on gene expression in thyrocytes. These results, taken together with those of iodide uptake, indicate that NO is able to inhibit thyroid function and repress thyroid-specific gene expression in cells long-exposed to variable large amounts of NO.

Effect of SNP on cGMP and cAMP production

As described above, we observed that NO could interfere with the stimulation exerted by TSH on differentiated thyroid cell functions. It is known that cGMP is one of the major

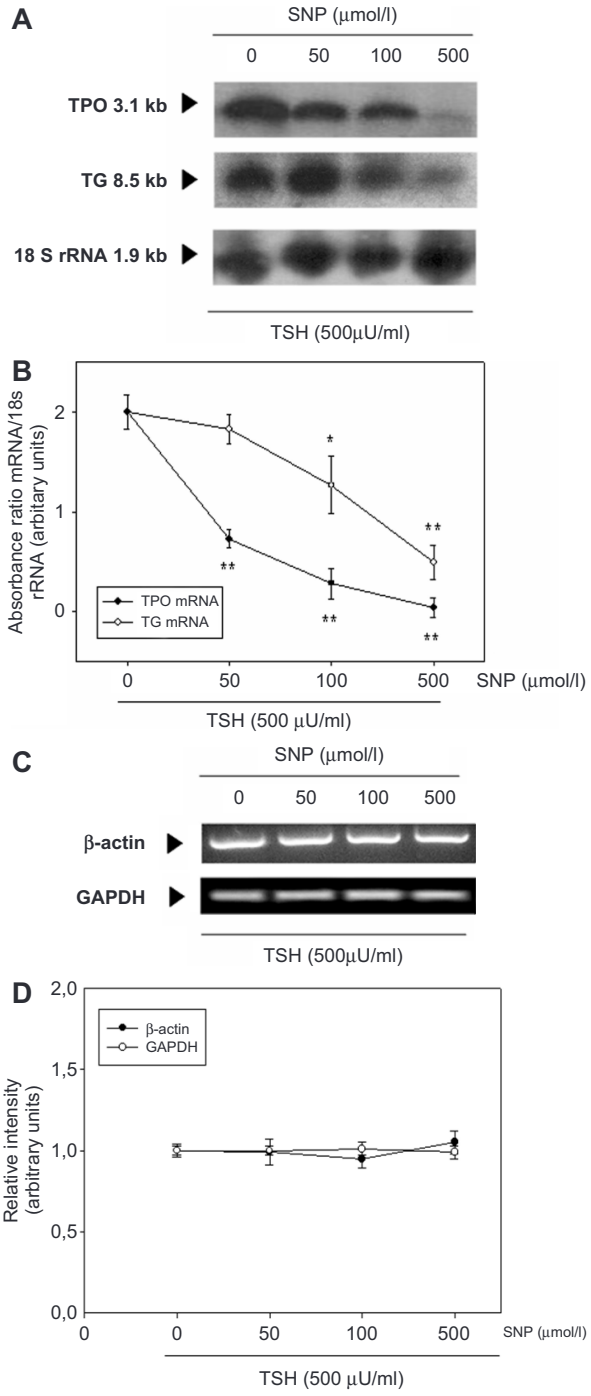

FIG. 2. Sodium nitroprusside (SNP) reduces the thyroid-stimulating hormone (TSH)–stimulated thyroperoxidase (TPO) and thyroglobulin (TG) mRNA levels. Basal FRTL-5 cells were incubated with TSH (500 μU/mL) in the presence or absence of SNP (50–500 μmol/L) for 24 h and then harvested for total RNA extraction. (**A**) Representative Northern blot hybridized with TPO, TG, and 18S ribosomal RNA (rRNA) recognizing probes. (**B**) Densitometric analysis of the Northern blots. Values were normalized to the 18S rRNA signal and expressed in arbitrary units. Each value represents the mean ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.001$ versus TSH alone (taken as 2.00). (**C**) Representative RT-PCR for β-actin and GAPDH. (**D**) Densitometric analysis of RT-PCR for β-actin and GAPDH (TSH alone taken as 1.00).

mediators of NO action in different cell types and that cAMP is the main mediator in the TSH regulation of thyroid function. Therefore, we investigated the simultaneous changes in the cyclic nucleotides production induced by SNP in the thyroid cell. We treated the cells for 24–48 h with SNP (10–500 μmol/L) in the presence of TSH (500 μU/mL) and measured the levels of cyclic nucleotides in the culture medium as an estimation of the intracellular production. As depicted in Figure 3A, SNP (50–500 μmol/L) increased the TSH-induced cGMP production at 24 and 48 h of incubation. The highest increment of cGMP was obtained with 500 μmol/L SNP at 48 h. Interestingly, TSH (500 μU/mL) significantly stimulated the production of cGMP at 24 and 48 h (Fig. 3A). When the cAMP level was analyzed in response to SNP treatment, we observed no significant changes respect to TSH alone with the lower SNP concentrations (Fig. 3B), whereas a significant decrease of cAMP production was detected with the highest SNP concentration (500 μmol/L) after 48 h of incubation (Fig. 3B). No changes in cGMP or cAMP levels were observed in basal cells treated with 500 μmol/L SNP (Fig. 3A, B). Thus, NO may mainly act increasing cGMP production without affecting cAMP level in thyroid cells. However, when high NO concentrations are in contact with thyroid cells for long periods of time, NO could also induce a reduction of the cAMP generation.

8-Br-cGMP inhibits the TSH-induced iodide uptake and thyroid-specific gene expression

To explore the role of cGMP as possibly involved in an inhibitory via on thyroid cell function, we analyzed the effect of 8-Br-cGMP, a cell membrane permeable analog of cGMP, on the TSH-induced iodide uptake as well as the TPO and TG mRNA expression. When the cells were incubated with 8-Br-cGMP (1000 μmol/L) for 24 h, we observed a significant inhibition of the TSH-stimulated iodide uptake (Fig. 4A). The effect was quantitatively similar to that exerted by SNP (50 μmol/L). The basal iodide uptake was not modified by 8-Br-cGMP (Fig. 4A). A reduction of the TSH-induced TPO and TG mRNA levels was observed after treatment of the cells with 8-Br-cGMP (300 and 1000 μmol/L) for 24 h (Fig. 4B). 8-Br-cGMP did not alter basal TPO or TG mRNA expression (data not shown). The ability of cGMP to mimic the SNP effects together with the above-described SNP-induced increase of cGMP level strongly suggests that cGMP could have a role in mediating the inhibitory action of NO on thyroid cells.

The cGK inhibitor, KT-5823, reverses the NO-induced reduction of the TSH-stimulated iodide uptake

To study whether a cGK-dependent mechanism was involved in the NO/cGMP-induced inhibition of iodide uptake, the action of the cGK inhibitor, KT-5823, was assayed. This agent was used at concentrations assumed in the literature to specifically inhibit cGK (20,34,35). TSH-stimulated cells were incubated with SNP (50 μmol/L) or 8-Br-cGMP (1000 μmol/L) in the presence of KT-5823 (10 μmol/L) for 24 h. It was observed that coincubation with KT-5823 reversed the SNP– or 8-Br-cGMP–inhibited iodide uptake values (Fig. 5). Similar results were obtained with 1 μmol/L KT-5823 (data not shown). Treatment with KT-5823 alone did not modify iodide uptake compared with basal values (data not shown).

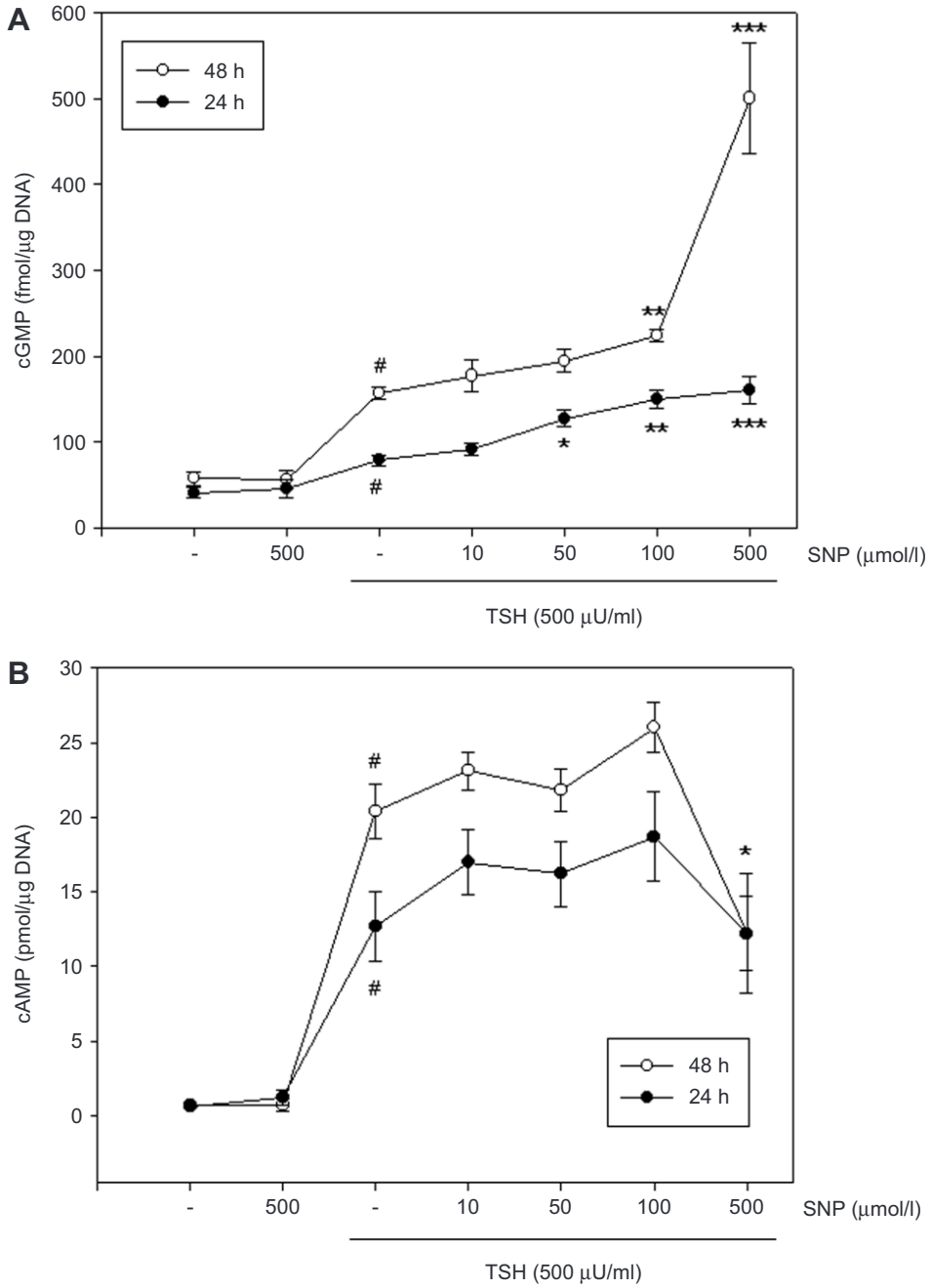

FIG. 3. Effect of sodium nitroprusside (SNP) on cyclic nucleotides production. Basal FRTL-5 cells were incubated with SNP (500 μmol/L) or with thyroid-stimulating hormone (TSH) (500 μU/mL) in the presence or absence of SNP (10–500 μmol/L) for 24 and 48 h. The 3′,5′-cyclic guanosine monophosphate (cGMP) (**A**) or cAMP (**B**) levels were determined in the culture medium. Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus TSH alone; $^{\#}p < 0.001$ versus basal (without TSH).

These results suggest that the cGMP/cGK pathway could be involved in the NO-induced inhibition of iodide uptake.

Effect of SNP on iodide uptake in cells pretreated with TSH

Since NO seems to interfere with the TSH action on parameters of thyroid hormone biosynthesis, we evaluated the effect of SNP (10–500 μmol/L) in cells pretreated with TSH for 24 and 48 h. As depicted in Figure 6A, SNP added to the cultured medium for 24 h after TSH pretreatment was not able to alter the TSH-induced iodide uptake, although an inhibitory effect on iodide uptake was exerted by SNP at the highest concentration (500 μmol/L) when it was incorporated after 48 h of pretreatment with TSH (Fig. 6A). The nitrite level induced by SNP was not modified at any time of TSH pretreatment (Fig. 6B), indicating that the blockage of SNP action by the TSH pretreatment is not a consequence of changes in the SNP-induced NO generation. To analyze whether lower TSH concentrations were able to prevent the inhibitory effect

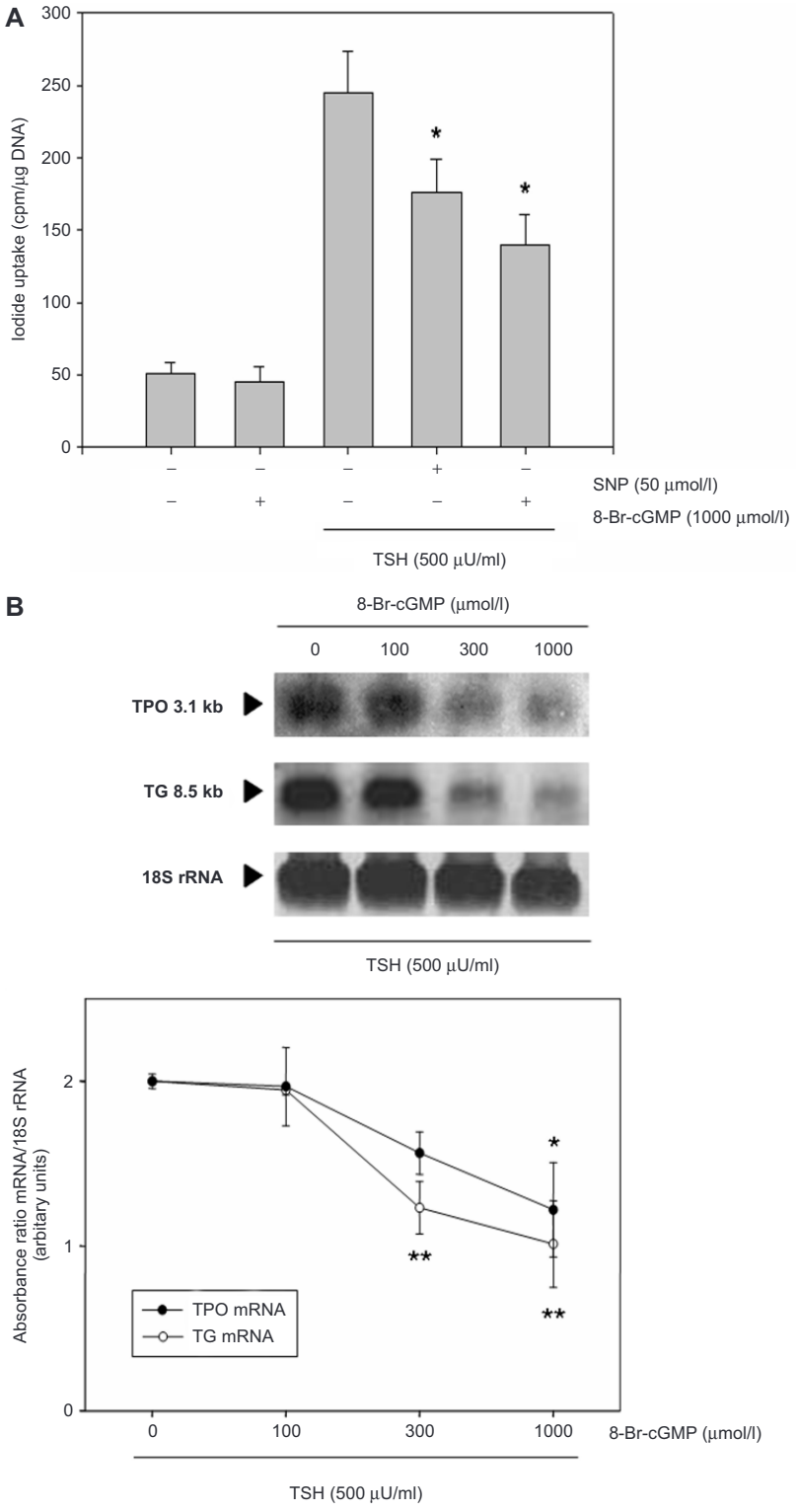

FIG. 4. 8-Bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) inhibits the thyroid-stimulating hormone (TSH)–induced iodide uptake and thyroperoxidase (TPO) and thyroglobulin (TG) mRNA expression. (**A**) Basal FRTL-5 cells were incubated with TSH (500 μU/mL) in the presence or absence of 50 μmol/L sodium nitroprusside (SNP) or 1000 μmol/L 8-Br-cGMP for 24 h. Values are expressed as counts per min (cpm)/μg DNA. Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. *$p < 0.01$ versus TSH alone. (**B**) Basal FRTL-5 cells were incubated with TSH (500 μU/mL) in the presence or absence of 8-Br-cGMP (100–1000 μmol/L) for 24 h and then harvested for total RNA extraction. Representative Northern blot hybridized with TPO, TG, and 18S ribosomal RNA (rRNA) recognizing probes (upper panel). Densitometric analysis of Northern blots. Values were normalized to the 18S rRNA signal and expressed in arbitrary units. Each value represents the mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ versus TSH alone (taken as 2.00) (lower panel).

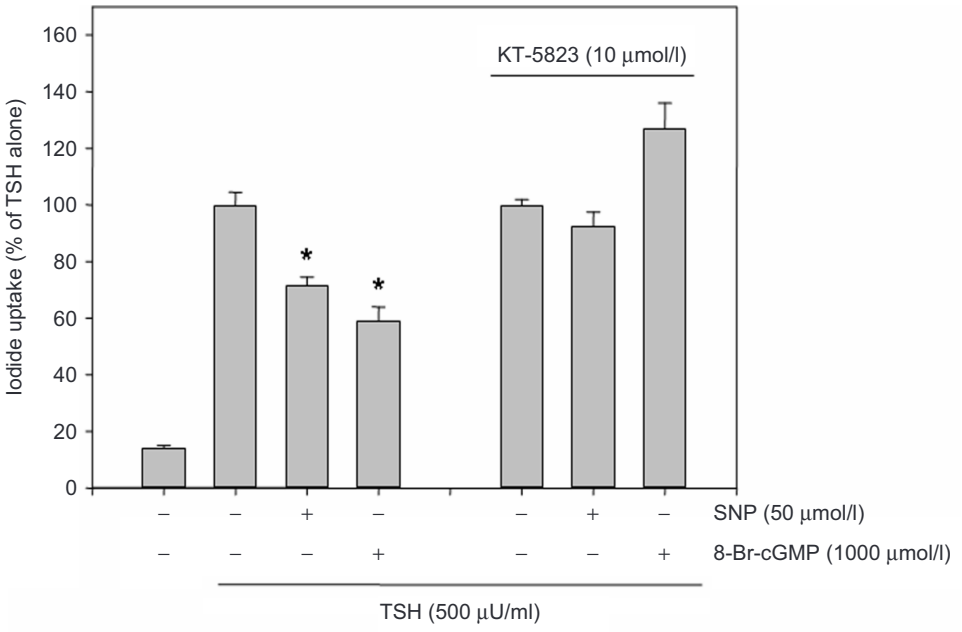

FIG. 5. KT-5823 reverses the sodium nitroprusside (SNP)–induced and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP)–induced inhibition of the thyroid-stimulating hormone (TSH)–stimulated iodide uptake. Basal FRTL-5 cells were incubated with TSH (500 μU/mL), TSH plus 50 μmol/L SNP, TSH plus 1000 μmol/L 8-Br-cGMP, TSH plus 50 μmol/L SNP in the presence of 10 μmol/L KT-5823, or TSH plus 1000 μmol/L 8-Br-cGMP in the presence of 10 μmol/L KT-5823 for 24 h. Values are expressed as percentage of TSH alone value (taken as 100%). Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. *$p < 0.01$ versus TSH alone.

of SNP, iodide uptake was evaluated in cells pretreated with 100 or 200 μU/mL TSH for 24 h and incubated with 100 μmol/L SNP for additional 24 h. After preincubation with any of the TSH concentrations, SNP was unable to inhibit the TSH-stimulated iodide uptake (Fig. 6C). We corroborated that SNP inhibited iodide uptake at all the TSH concentrations assayed in cells without TSH pretreatment (Fig. 6C). The stimulation of iodide uptake at the different TSH concentrations was rather similar although a slightly higher increase was obtained with 500 μU/mL TSH (Fig. 6C). These observations appear to indicate a competition among the intracellular signals produced by TSH and those induced by NO, the resultant effect being dependent on the degree of the TSH and/or NO stimulus.

Discussion

The present study demonstrates that the NO released from the NO donor SNP exerts an inhibitory effect on several markers of thyroid differentiation in FRTL-5 cells. In accordance, previous observations indicated that NO donors reduced iodide uptake in calf (20) and iodide organification in human (17) thyroid follicles in culture after 2–4 h of incubation. At longer times (24–96 h), NO donors inhibited iodide transport and organification, and induced morphological changes in cultured bovine thyroid cells (21). In contrast, SNP did not modify triiodothyronine secretion in cultured human thyrocytes (23). The experimental model of continuous well-differentiated thyroid cell line FRTL-5 here used has stable functional characteristics and, unlike primary cultures, allows to eliminate possible effects arising from nonfollicular cells (25,26). Therefore, here we reveal the property of NO to reduce thyroid-differentiated functions by a direct effect on thyroid follicular cells. In agreement, we recently reported that the thyrocyte-produced NO could act as a negative signal in the regulation of thyroid cell function (24). Consequently, 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 our experimental conditions the nitrite accumulation after metabolization of SNP paralleled the NO donor–induced inhibition of thyroid function, suggesting that NO is responsible for the inhibitory effect. The involvement of a toxic action could be discarded because of the conserved cell viability after SNP treatment here observed and the reversibility of its inhibitory action on functional parameters previously reported in bovine thyroid cells (20,21).

The present results reveal a novel negative action of NO on thyroid-specific gene expression in thyrocytes. This agrees with the recent report of a partial mediation of NO in the inhibitory effect of interleukin 1 alpha (IL-1α)/interferon gamma (IFN-γ) on TPO and thyroid oxidases (ThOXs) expression in human thyrocytes (19). The reduction of TPO and TG mRNA expression by NO could explain the NO donor–induced long-term inhibition of iodide organification previously reported (21). However, the decrease of iodide uptake in calf (20) and organification in human (17) thyroid cells after few hours of incubation with NO donors is in favor of posttranslational effects of NO. Since iodide organification in FRTL-5 cells is very low, the NO-induced inhibition of iodide uptake here demonstrated seems to involve a reduced iodide transport. This is in agreement with the inhibition of iodide transport by NO previously observed in calf (20) and bovine (21) thyroid cells, supporting that the NO action could be exerted at different levels on thyroid hormone biosynthesis.

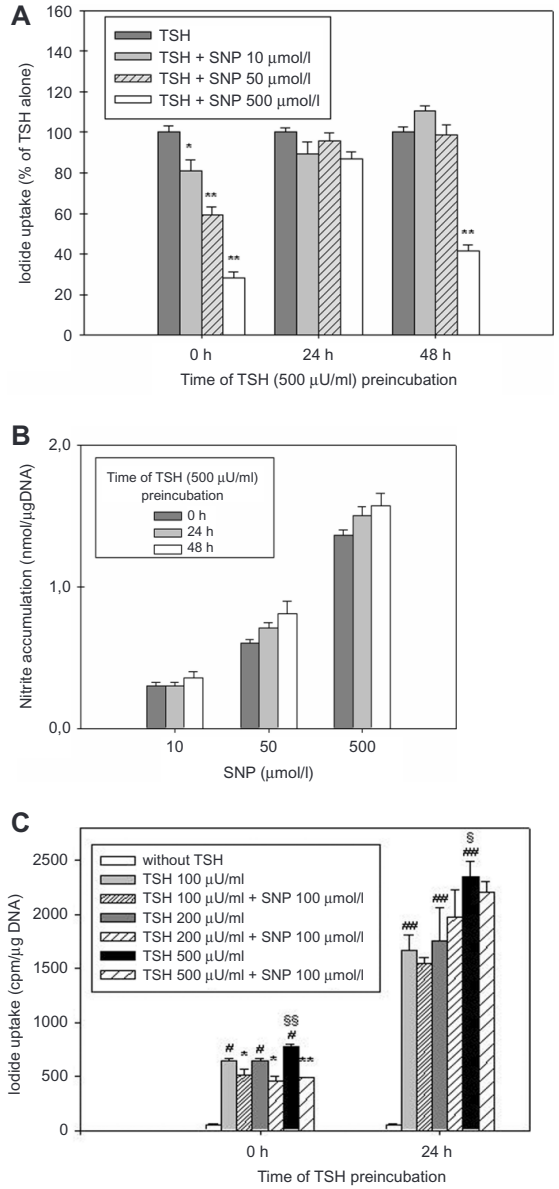

FIG. 6. Sodium nitroprusside (SNP) action on iodide uptake in cells pretreated with thyroid-stimulating hormone (TSH). (**A**) Basal FRTL-5 cells were incubated with TSH (500 μU/mL) for 24 and 48 h, and then SNP (10–500 μmol/L) was added to the culture medium and incubation continued for additional 24 h. Iodide uptake is expressed as percentage of the iodide uptake in cells incubated with TSH alone (taken as 100%). Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. $^{*}p < 0.01$ and $^{**}p < 0.001$ versus TSH alone. (**B**) Nitrite level in the culture medium of SNP-treated cells preincubated with TSH as described in (**A**). Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. (**C**) Basal FRTL-5 cells were incubated with TSH (100–500 μU/mL) for 24 h, and then SNP (100 μmol/L) was added to the culture medium for additional 24 h. Iodide uptake is expressed as counts per min (cpm)/μg DNA. Data represent the mean ± SEM of three independent experiments in which quadruplicate samples were analyzed. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus TSH alone; $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ versus without TSH; $^{\S}p < 0.05$ versus 100 μU/mL TSH; $^{\S\S}p < 0.01$ versus 100 and 200 μU/mL TSH.

Previous evidence has involved cGMP as one of the main mediators of the NO effects on cell functions (3,4,8). In accordance, we observed a time- and dose-dependent elevation of cGMP in response to SNP, which suggests the activation of the soluble guanylyl cyclase/cGMP pathway by NO. These findings agree with previous reports indicating that SNP increased the cGMP production in dog (13), human (23), and calf (20) thyroid cells. Our observation that the cell membrane permeable cGMP analog, 8-Br-cGMP, exerts an inhibitory action similar to that of NO on iodide uptake and thyroid-specific gene expression is in favor of a role of cGMP in the NO action. In contrast, it has been reported that a cGMP analog had no effect on iodide organification in human thyroid follicles (17), and several studies have observed a cGMP-mediated stimulation of some functional parameters in thyroid cells (36). However, other studies coincide in demonstrating a cGMP-mediated inhibition of different steps of thyroid hormone biosynthesis (20,21,37). Here we report a new ability of cGMP to reduce TPO mRNA expression in thyrocytes and reinforce the ability of cGMP to inhibit the TG mRNA expression previously observed in bovine thyroid cells (37), favoring a role of cGMP in the regulation of thyroid-expressed genes.

It is well known that TSH influences virtually all aspects of differentiated thyroid cell function through cAMP signaling (36). In this study we found that long-time exposition of FRTL-5 cells to SNP did not modified cAMP production except at high dose. Similarly, it has been observed that TSH-stimulated cAMP level was not modified after treatment with SNP for 10–20 min in dog thyroid (22) or for 2 h in human thyrocytes (23). According to our results, the reduction in the cAMP production could be relevant in the inhibitory action of relatively high levels of NO for a long time. It is not clear whether the SNP-induced cAMP decrease could be caused by the increased cGMP level. Thus, it is known that cGMP regulates several 3′,5′-cyclic nucleotide phosphodiesterases that hydrolyze cAMP (4,22). Nevertheless, since we observed that moderate SNP concentrations increased cGMP but did not modify cAMP levels, a cGMP-induced reduction of cAMP could be a minor mechanism in the NO action on the thyroid cell. In agreement, the decrease in cAMP accumulation induced by carbamylcholine in TSH-stimulated dog thyroid slices could not be attributed to the enhanced cGMP level produced by the drug (22). Our results support that the NO/cGMP-mediated inhibitory signal on thyroid hormone biosynthesis could be hardly related to a cAMP reduction in FRTL-5 thyroid cells.

The fact that TSH increased cGMP levels in our experimental system suggests an activation of guanylyl cyclase by TSH in the thyroid cell. It is possible that TSH could activate the enzyme activity through NO production. Supporting this supposition, we previously observed that TSH was able to induce NO generation in bovine thyrocytes (21). Contrarily, TSH did not stimulate NO production in human thyrocytes (17). Although in the present study it could not be detected a TSH-induced NO production as nitrite accumulation probably due to a low sensitivity of the colorimetric assay, we could measure a TSH-stimulated NO generation by a radiometric analysis in FRTL-5 cells (24). In contrast to our results, no change of the cGMP level after TSH treatment was reported in dog (38), human (23,39,40), and calf (20) thyroid cells. However, cyclic GMP levels showed a large and rapid increase after TSH stimulation in rat thyroid follicular cells (41). Such

apparent discrepancies could be explained by differences in species or experimental procedures although they might be indicating that the stimulation of cGMP by TSH could take place in certain conditions of thyroid cell activity. Since cGMP has been involved in inhibitory pathways in thyroid cells by the present and previous reports (20,21,24,37), the TSH-induced increase of cGMP here observed in FRTL-5 cells seems to oppose the stimulating effect of TSH. In accordance, we have provided evidence that a TSH-dependent endogenous NO production by thyrocytes could act as a negative signal in thyroid cell regulation (24).

The reversal of the SNP– or 8-Br-cGMP–induced reduction of iodide uptake by the cGK inhibitor KT-5823 that we observed suggests that the activation of cGK is implicated in the inhibitory action of NO/cGMP on iodide uptake in FRTL-5. In accordance, an inhibitory effect of SNP on iodide uptake and its reversion by KT-5823 have been previously shown after 3 h of SNP exposition in bovine thyroid cells (20). The lack of effect of KT-5823 on TSH-stimulated iodide uptake obtained in our experimental conditions does not support a relevant inhibition of iodide uptake by the TSH-induced cGMP production. However, we previously observed that KT-5823 was able to increase the iodide uptake stimulated by a lower TSH concentration for a longer time in FRTL-5 cells (24).

The reduction of the inhibitory action of SNP on iodide uptake in cells previously treated with TSH seems to indicate a less efficient action of NO when the TSH-induced signaling pathways have been previously stimulated. Interestingly, it appears to be in accordance with the lack of the NO-mediated inhibition of TPO and ThOXs expression by cytokines observed in thyrocytes from autonomous toxic nodules as well as in normal thyrocytes treated with high concentrations of TSH. This phenomenon was suggested to involve a partial resistance of hyperstimulated thyroid cells with an activated cAMP pathway to the NO-mediated inhibitory effect of cytokines (19).

It has been observed that NOS II and NOS III expression was increased in human thyroid tumors and autoimmune lesions (15,16). A mediation of NO in the cytokine-induced cytotoxic effects and functional injuries on follicular cells in autoimmune thyroiditis has been proposed (18,19). The long-term inhibition of steps of thyroid hormonogenesis by NO here observed could be of interest in relation to human thyroid pathologies associated with a chronic NO production.

In summary, the present study supports a long-term inhibitory role of the NO/cGMP pathway on TSH-stimulated parameters of thyroid hormone biosynthesis in FRTL-5 thyroid cells. It is suggested that the activation of cGK by cGMP could be a mechanism involved in the inhibitory effect of NO on iodide uptake. Evidence for the involvement of the NO/cGMP signaling in the regulation of thyroid-specific gene expression is provided. These findings favor inhibitory actions of NO on thyroid cell function that could be of pathophysiological relevance.

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