Endogenous thyrocyte-produced nitric oxide inhibits iodide uptake and thyroid-specific gene expression in FRTL-5 thyroid cells

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Abstract

Nitric oxide (NO) is a free radical that mediates a wide array of cell functions. It is generated from L-arginine by NO-synthase (NOS). Expression of NOS isoforms has been demonstrated in thyroid cells. Previous reports indicated that NO donors induce dedifferentiation in thyrocytes. However, the functional significance of endogenous thyrocyte-produced NO has not been explored. This work aimed to study the influence of endogenous NO on parameters of thyroid cell function and differentiation in FRTL-5 cells. We observed that treatment with the NOS inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME), increased the TSH-stimulated iodide uptake. The TSH-induced sodium iodide symporter (NIS) and thyroglobulin (TG) mRNA expressions were increased after incubation with L-NAME. In transient

transfection assays, TSH-stimulated transcriptional activities of NIS and TG promoters were increased by L-NAME. An increment of the TSH-stimulated cell proliferation was observed after NOS inhibition. Similar results were obtained when the action of another NOS inhibitor, N $^{\epsilon}$ -monomethyl-L-arginine, was analysed for most of these studies. The production of NO, which was not detectable in basal conditions, was increased by TSH. Our data provide strong evidence that endogenous NO could act as a negative signal for TSH-stimulated iodide uptake and thyroid-specific gene expression as well as proliferation in thyrocytes. These findings reveal a possible new inhibitory pathway in the regulation of thyroid cell function.

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Introduction

Nitric oxide (NO) is a versatile molecule with a wide spectrum of effects in several tissues (Laskin *et al.* 1994, Guix *et al.* 2005). The synthesis of NO is catalysed by NO synthase (NOS). Three isoforms of NOS (I–III) have been well characterized, which are widely expressed in most cell types. The NOS-mediated reaction involves L-arginine oxidation with generation of NO and L-citrulline (Nathan & Xie 1994, Guix *et al.* 2005). One of the physiologically more relevant targets of NO is the soluble guanylyl cyclase. The binding of NO to this enzyme leads to the formation of 3',5'-cyclic guanosine monophosphate (cGMP), which is involved in the activation of a variety of effectors including cGMP-dependent protein kinase (cGK; Krumenacker *et al.* 2004).

Thyroid hormone biosynthesis involves iodide uptake by the sodium iodide symporter (NIS) with later iodination of thyroglobulin (TG) by the action of thyroid peroxidase (TPO). All these events are regulated by thyrotrophin (TSH) mainly by the modulation of thyroid-specific genes, such as NIS, TG and TPO (Dunn & Dunn 2001).

The existence of a basal tonic level of endogenous NO generation with a possible autocrine or paracrine role in the

thyroid tissue has been proposed (Esteves *et al.* 1992). Thus, evidence has been obtained for a cGMP increase mediated by endogenous NO in dog thyroid slices (Esteves *et al.* 1992). The three isoforms of NOS have been detected in the rat thyroid gland being the greatest level of basal expression displayed by NOS III (Colin *et al.* 1995). It has been demonstrated that NOS III is expressed in the thyroid follicular cell (Colin *et al.* 1997).

Several effects of NO on thyroid function have been demonstrated using NO donors. We previously reported that the NO donors, sodium nitroprusside (SNP) and S-nitrosoglutathione, inhibited iodide uptake, organification and transport in bovine thyroid cells (Costamagna *et al.* 1998). In accordance, SNP reduced iodide uptake in primary cultures of calf thyrocytes (Bocanera *et al.* 1997). It has been reported that SNP increased the cGMP production in dog (Esteves *et al.* 1992), human (Millatt *et al.* 1993) and calf (Bocanera *et al.* 1997) thyroid cells. The NO generation induced by NO donors would resemble what might occur in some abnormal conditions of thyroid tissue in which increased levels of NO could contact with thyrocytes. Nevertheless, the functional significance of the endogenous thyrocyte-generated NO has not been examined.

In several cell types, the role of endogenous NO production has been extensively studied by manipulation of biological activity of NOS with NOS inhibitors. NOS is competitively inhibited by L-arginine analogues including N ω -nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) (Alderton *et al.* 2001, Albrecht *et al.* 2003). In the present study, we analysed the significance of the endogenously generated NO on the regulation of some TSH-stimulated parameters of function, differentiation and proliferation by inhibition of NOS with L-NAME and L-NMMA. The effect of TSH on NO production in the thyroid cell was also examined.

Materials and Methods

Cell culture

FRTL-5 rat thyroid cells (ATCC CRL 8305; American Type Culture Collection) were kindly provided by Dr L Kohn (Edison Biotech Institute, Ohio University, USA) and had the properties reported previously (Ambesi-Impiomato *et al.* 1980). Cells were grown in a 5% CO₂–95% air atmosphere in Coon's modified Ham F-12 medium (DMEM/Ham F-12, 1:1), supplemented with 5% calf serum (PAA Laboratories GmbH, Linz, Austria), 1 mIU/ml bovine TSH (a generous gift of the NIDDK National Pituitary Hormone Program & Dr AF Parlow, NIH, Baltimore, MD, USA), 10 µg/ml bovine insulin, 5 µg/ml bovine transferrin, 2 µmol/ml glutamine, antibiotics and an antimycotic (Sigma). Cells were passaged every 7–8 days and fed fresh medium every 2–3 days. When they reached 70% of confluence, the cells were shifted to medium without TSH (basal medium) containing 0.2% calf serum and maintained for 5–7 days before the experiments (Vélez *et al.* 2006). Starved (basal) cells were treated with the indicated concentration of TSH in the presence or absence of the NOS inhibitors L-NAME (1 mM) or L-NMMA (0.1 mM; Sigma) or the cGK inhibitor KT-5823 (5 µM) (Calbiochem, San Diego, CA, USA) for different periods of time. All cultured cells were used before passage 20.

Iodide uptake

The iodide uptake assay was performed as described (Costamagna *et al.* 1998) with minor modifications. Briefly, FRTL-5 cells seeded in 24-well tissue culture plates were incubated with 0.1 Na¹³¹I (carrier-free) and 1 µmol/l KI in Hank's Balanced Salt Solution (HBSS) for 35 min at 37 °C. Thereafter, cells were washed, scraped and centrifuged at 1500 g for 10 min. Iodide uptake was determined by measuring the total radioactivity incorporated into the cells and expressed as counts/minute per microgram DNA.

DNA content analysis

The DNA content was measured in the cellular pellet by the diphenylamine method of Burton (1956) modified by Kohn & Valente (1989).

RNA isolation

Total RNA was purified from FRTL-5 cells by the acid guanidinium thiocyanate/phenol/chloroform extraction procedure of Chomczynski & Sacchi (1987). Briefly, the cells were harvested in a denaturing solution containing 4 M guanidine thiocyanate. The suspension was mixed sequentially with 2 M 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.

Northern blot analysis

Total RNA (20 µg) was subjected to electrophoresis on 1% agarose gels containing 0.66 M formaldehyde (Sigma). RNA was transferred to nylon membranes (Sigma) by capillary transblotting overnight. Membranes were baked for 2 h at 80 °C and stained with methylene blue to reveal the integrity of RNA and sample loading. Membranes were hybridized overnight at 42 °C in a solution containing α -³²P-ATP-probes labelled by random primer methodology (Sambrook *et al.* 1989). Rat TG (Di Lauro *et al.* 1982) and NIS (Dai *et al.* 1996) cDNAs were used as probes. Blots were washed in 2×SSC/1% SDS at room temperature, twice with each one in 2×SSC/1% SDS and 0.2×SSC/1% SDS at 55 °C. Then, the membranes were exposed to Kodak X-Omat film at –80 °C and band intensities determined by scanning densitometry (Scion Image software, Scion Corporation, Fredrick, MD, USA).

Semiquantitative RT-PCR

cDNA was synthesized from 1 µg total RNA. A semiquantitative multiplex RT-PCR was designed to compare the RT-PCR products of the NIS gene with those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to determine their relative expression levels. PCR was performed in a 20 µl reaction volume containing 2 µl cDNA reaction, 0.5 µM of each primer, 0.25 mM of each dNTP, 1.5 mM MgCl₂ and 0.8 units Taq polymerase. The mixed samples were heated to 95 °C for 4 min and then subjected to 29 cycles of amplification performed as follows: denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, followed by a 7 min extension at 72 °C after the last cycle. Primer oligonucleotides for the rat NIS gene were: 5'-GCTGTGGCATTGTTCATGTTTC (forward) and 5'-TGAGGTCTTCCACAGTCACA (reverse). The amplification yielded a 219 bp DNA product whose

sequence corresponded to that of fragment 1002–1220 of the rat NIS gene (Dai *et al.* 1996). The identity of the NIS amplicon was confirmed by automatic sequencing analysis. Primer oligonucleotides for the rat GAPDH gene were: 5′-GAGTATGTCGTGGAGTCTACTG (forward) and 5′-GCTTCACCACCTTCTTGATGTC (reverse). The amplification yielded a 514 bp DNA product corresponding to fragment 1120–1633 of the rat *GAPDH* gene, as reported in the Gene Bank (Accession number AF106860). To ensure that the amplification of both rat NIS and rat GAPDH remained within the exponential range, reaction conditions were optimized by assessing the variation in signal intensity for the two genes at various sample amounts and cycle numbers. Three different primer ratios were tested to achieve the same efficiency of amplification, and the ratio 1:1 (NIS:GAPDH) was selected. The primers of rat GAPDH were added after the first 10 cycles of the reaction. Genomic DNA contamination was excluded since extra bands were not observed although the primer pairs used for NIS and GAPDH spanned introns. As negative control, reactions without reverse transcription were carried out in parallel. PCR products were separated on 1.5% agarose gel and visualized with ethidium bromide. The bands of a positive film were scanned, and the density and width of each band measured using the Scion Image software. All primers were from Sigma-Genosys.

Promoter constructs

pTG Luc construct contained the minimal wild-type promoter of the rat TG gene (−168 to +36 bp) linked to luciferase (Luc) reporter gene cloned in the pGL2-Basic plasmid (Kang *et al.* 2001) and pNIS Luc corresponded to the NIS promoter region from −2841 to +13 bp linked to Luc gene cloned in the pBSLUC2 plasmid (Garcia & Santisteban 2002). As negative control, the promoterless pGL3-Basic (Promega) was used. The cytomegalovirus promoter linked to β-galactosidase gene plasmid (CMV-β gal) (Promega) was used to monitor transfection efficiency.

Transfection assays

FRTL-5 cells were plated at a density of 6×10^5 per 60 mm diameter tissue culture dish 48 h before transfection. The calcium phosphate DNA co-precipitation method was used for transfections as previously described (Vélez *et al.* 2006). Cells were incubated with the precipitate containing 4 µg construct of interest and 0.8 µg CMV-β gal. Transfected cells were cultured in basal medium for 5 days and then treated with TSH in the presence or absence of NOS inhibitors. After the indicated time, the cells were harvested in PBS solution, centrifuged and resuspended in passive lysis buffer (Promega). Lysates were centrifuged, and the supernatants assayed for Luc and β gal activities as described (Brasier *et al.* 1989, Endo *et al.* 1997).

[³H]Thymidine incorporation

The [³H]thymidine incorporation measurement was performed as described previously (Ambesi-Impimbato *et al.* 1980) with minor modifications. FRTL-5 cells were seeded in 96-well plates at a density of 8×10^3 cells/well and when they reached approximately 40% confluence they were shift to basal medium for 4 days. Then, TSH was added alone or in combination with NOS inhibitors in the presence of [³H]thymidine (1 µCi/well) for 24–48 h at 37 °C in a 95% air–5% CO₂ atmosphere. After incubation, cells were washed and harvested onto glass fibre filters (Whatman 934-4-H, Whatman Paper Ltd, Maidstone, UK) and the radioactivity incorporated into the dried cells was evaluated by liquid scintillation counting. All determinations were made in quadruplicate and expressed as counts per minute [³H]thymidine/well.

CFSE-based proliferation assay

FRTL-5 cells were cultured in basal medium for 2 days and then labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described (Wang *et al.* 2005) with modifications. Briefly, cells cultured in basal medium were harvested, centrifuged and resuspended in PBS. The 5 mM CFSE stock solution (Cat. no. C-1157, Molecular Probes, Carlsbad, CA, USA) was diluted in PBS containing 5% of fetal bovine serum (FBS) in a total volume of 1 ml. CFSE was added to the cell suspension at 7.5 µM final concentration and incubated for 30 min at room temperature. The labelled cells were washed twice with basal medium supplemented with 5% FBS, plated at a density of 3×10^6 per 100 mm diameter tissue culture dish in basal medium for 3 additional days and then treated with TSH with or without NOS inhibitors. Data were acquired on a Cytoron Absolute cytometer (Ortho Diagnostic System, Raritan, NJ, USA) and analysed using WinMDI 2.8 software (J Trotter, Scripps Institute, La Jolla, CA, USA).

NO production

Cells were loaded with L-[¹⁴C]arginine and the amount of L-[¹⁴C]citrulline generated was quantified to estimate NO production by a modification of the previously described technique (Grider 1993). As L-citrulline and NO are produced in a 1:1 ratio from L-arginine by the action of NOS, L-citrulline synthesis represents a sensitive marker of NO formation. L-[¹⁴C]arginine (0.1 µCi) was added to the culture medium for 30 min at 37 °C. Then the cells were scraped and centrifuged for 10 min. Supernatants were transferred to 1 ml columns of Dowex AG50W-X8 (Na⁺ form) and the [¹⁴C]citrulline was eluted with water. The radioactivity corresponding to the [¹⁴C]citrulline in the eluate was measured by liquid scintillation counting and normalized by micrograms of DNA. Results were expressed as percentage increase above the basal levels measured in cells cultured in the absence of TSH.

Statistical analysis

Analysis of multiple inter-group differences was conducted by one-way ANOVA. As post-test, the Student–Newman–Keuls multiple comparisons test was used. Differences were considered significant at $P < 0.05$.

Results

NOS inhibition increased the TSH-stimulated iodide uptake

To study the possible involvement of the endogenously produced NO on the regulation of iodide uptake, a crucial step in thyroid hormone biosynthesis, cells were treated with the NOS inhibitor, L-NAME. As expected, TSH (100–1000 $\mu\text{IU/ml}$) stimulated iodide uptake in FRTL-5 cells (Fig. 1A). The incubation of cells

Figure 1 L-NAME treatment increased the TSH-stimulated iodide uptake. (A) Basal FRTL-5 cells were treated with TSH (100–1000 $\mu\text{IU/ml}$) alone or in the presence of 1 mM L-NAME for 48 h. Values were expressed as counts/min per mg DNA. (B) Basal FRTL-5 cells were treated with TSH (200 $\mu\text{IU/ml}$) with or without 1 mM L-NAME for 24, 48 and 72 h. Data are expressed as percentage of TSH alone value (taken as 100%). Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$, † $P < 0.01$ vs TSH; ‡ $P < 0.05$, § $P < 0.01$ vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

with L-NAME (1 mM) in the presence of TSH for 48 h significantly increased the TSH-induced iodide uptake (Fig. 1A). Since the maximal stimulation of iodide uptake was observed with 200 $\mu\text{IU/ml}$ TSH, this concentration of TSH was used for time-course experiments and to analyse the other functional parameters. An increase of the TSH-induced iodide uptake was observed in the presence of L-NAME at 24, 48 and 72 h (Fig. 1B). Treatment with L-NAME (1 mM) alone did not modify iodide uptake when compared with basal values (Fig. 1A). These findings seem to indicate that endogenous NO exerts an inhibitory effect on TSH-stimulated iodide uptake.

The cGK inhibitor, KT-5823, increased the TSH-stimulated iodide uptake

To investigate whether a cGK-dependent mechanism is involved in the NO-induced inhibition of iodide uptake, the action of a specific cGK inhibitor, KT-5823 was assayed. Basal FRTL-5 cells were incubated with TSH (200 $\mu\text{IU/ml}$) in the presence of 5 μM KT-5823 for 48 h. As shown in Fig. 2, KT-5823 significantly increased the TSH-stimulated iodide uptake, suggesting the involvement of the cGMP/cGK pathway in the effect of NO. Treatment with KT-5823 alone did not modify iodide uptake when compared with basal values (Fig. 2).

NOS inhibition increased the TSH-induced NIS mRNA expression

In order to further explore the molecular mechanism involved in NO action on iodide uptake, NIS mRNA level was analysed by northern blot. A single NIS transcript (2.8 kb) was revealed. We found that the treatment of cells with L-NAME increased the TSH (200 $\mu\text{IU/ml}$)-stimulated NIS mRNA level at 48 h (Fig. 3A). Densitometric analysis of northern blot indicated that NIS mRNA was significantly

Figure 2 KT-5823 increased the TSH-stimulated iodide uptake. Basal FRTL-5 cells were treated with TSH (200 $\mu\text{IU/ml}$) alone or in the presence of 5 μM KT-5823 for 48 h. Values were expressed as counts/min per mg DNA. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ vs TSH; † $P < 0.05$ vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

Figure 3 L-NAME treatment increased TSH-induced NIS mRNA expression. Basal cells were treated with 200 µIU/ml TSH alone or in the presence of 1 mM L-NAME for 48 h. (A) Representative northern blot (20 µg) hybridized with rat NIS cDNA probe (upper panel). (B) Densitometric analysis of northern blot. Values were normalized to 18S rRNA. (C) Representative NIS RT-PCR. The lower bands represent NIS-specific product (219 bp) and the upper bands GADPH product (514 bp). Lanes represent: 1, molecular weight marker (100 bp ladder); 2, basal cells (without TSH); 3, 200 µIU/ml TSH alone; 4, 200 µIU/ml TSH+L-NAME; 5, no reverse transcription; 6, reverse transcription without RNA. (D) Densitometric analysis of NIS RT-PCR. Data are expressed as the level of NIS mRNA relative to GADPH mRNA. Each value represents the mean $\pm$ S.E.M. of three independent experiments. Basal value was taken as 1.00. ^{*} $P < 0.01$ vs TSH alone; [†] $P < 0.05$ vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

augmented by L-NAME (Fig. 3B). The increase of NIS mRNA level induced by L-NAME was verified by semiquantitative RT-PCR under similar conditions of cell treatment. A specific PCR product was detected in all samples analysed (Fig. 3C). In accordance with northern blot results, RT-PCR analysis revealed that L-NAME produced a significant increase of TSH-induced NIS mRNA (Fig. 3D). Treatment with L-NAME (1 mM) alone did not modify NIS mRNA level in northern blot or RT-PCR when compared with basal values (data not shown). These findings were in accordance with the stimulation of the TSH-induced iodide uptake observed after NOS blockage and suggested that the NO-mediated inhibition of iodide uptake could involve a reduction of NIS expression.

NOS inhibition increased the TSH-stimulated NIS promoter functional activity

To study the mechanism by which NO could inhibit NIS mRNA expression, we analysed the functional activity of the NIS promoter in the presence of the NOS inhibitor, L-NMMA. Transient transfection assays were performed with a Luc reporter gene under the regulation of the rat NIS promoter region from –2841 to +13 bp (pNIS Luc; Fig. 4A) (Garcia & Santisteban 2002). Figure 4B shows the stimulatory effect of TSH on pNIS Luc promoter activity as it is known (Garcia & Santisteban 2002). After incubation with L-NMMA for 24 h, we observed an increase of the TSH-stimulated pNIS Luc activity (Fig. 4B). Treatment with L-NMMA (0.1 mM)

Figure 4 L-NMMA increased the TSH-stimulated NIS promoter activity. (A) Schematic drawing of pNIS Luc containing the rat NIS promoter linked to Luc reporter gene. (B) FRTL-5 cells were transfected with 4 µg pNIS Luc and 0.8 µg CMV-βgal. After transfection, cells were maintained for 5 days in basal medium and then treated with TSH alone or in the presence of 0.1 mM L-NMMA for 24 h. Relative Luc/βgal activity is expressed as x-fold induction over the value of basal cells (taken as 1.00). Data represent the mean $\pm$ S.E.M. of at least three independent experiments. ^{*} $P < 0.01$ vs TSH alone; [†] $P < 0.05$ vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

alone did not modify pNIS Luc promoter activity when compared with basal values (Fig. 4B). Similar results were obtained using the other NOS inhibitor, L-NAME (data not shown). These results provide evidence for an inhibitory effect of NO on NIS promoter activity that may account for the reduced NIS mRNA levels induced by NO.

NOS inhibition increased the TSH-stimulated TG mRNA expression

We evaluated the effect of the endogenous thyrocyte-produced NO on the expression of TG, another key gene for normal thyroid function. It was observed that treatment of cells with L-NAME (1 mM) significantly increased the TSH-stimulated TG mRNA expression at 48 h (Fig. 5A and B). Similar results were obtained with other NOS inhibitor, L-NMMA (data not shown). These observations together with those obtained for NIS expression support a role of endogenous NO in controlling thyroid-specific gene expression.

NOS inhibition increased the TSH-stimulated TG promoter activity

In order to analyse whether the NO-mediated negative effect on TG mRNA expression could involve a

Figure 5 L-NAME treatment increased TSH-stimulated TG mRNA expression. Basal cells were treated with 200 µU/ml TSH alone or in the presence of 1 mM L-NAME for 48 h. (A) Representative northern blot (20 µg) hybridized with rat TG cDNA probe (upper panel) and 18S rRNA (lower panel) probes. (B) Densitometric analysis of northern blot. Values were normalized to 18S rRNA. Each value represents the mean $\pm$ S.E.M. of at least three independent experiments. Basal value was taken as 1.00. * P < 0.01 vs TSH alone; † P < 0.05 vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

transcriptional mechanism, we evaluated the TSH-induced TG promoter activity under endogenous NO inhibition. A Luc reporter construct containing the minimal rat TG promoter from –168 to +36 bp (pTG Luc; Fig. 6A) (Kang *et al.* 2001) was transiently transfected to FRTL-5 cells. We observed that the L-NMMA-induced NOS inhibition increased the TSH-stimulated transcriptional activity of pTG Luc at 12 and 24 h (Fig. 6B). Similar results were obtained in the presence of L-NAME (1 mM) for 24 h (Fig. 6C). Treatment with L-NAME (1 mM) or L-NMMA (0.1 mM) alone did not modify pTG Luc activity when

Figure 6 NOS inhibitors increased TSH-stimulated TG promoter activity. (A) Schematic drawing of pTG Luc containing the rat TG promoter linked to Luc reporter gene. (B) FRTL-5 cells were transfected with 4 µg pTG Luc and 0.8 µg CMV-βgal. After transfection, cells were maintained for 5 days in basal medium and then treated with 200 µU/ml TSH alone or with 0.1 mM L-NMMA for 12 and 24 h. (C) Cells transfected as indicated in B were treated with 200 µU/ml TSH with or without 1 mM L-NAME for 24 h. Relative Luc/βgal activity is expressed as x-fold induction over the value of basal cells (taken as 1.00). Data represent the mean $\pm$ S.E.M. of at least three independent experiments. * P < 0.05, † P < 0.01 vs TSH alone; * P < 0.05 vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

compared with basal values (Fig. 6B and C). These results provided evidence for an inhibitory effect of the endogenous NO on thyroid-specific gene expression at transcriptional level.

NOS inhibition increased the TSH-stimulated cell proliferation

It has been reported that NO regulates the growth of different cell types in a dual manner. We examined whether the endogenously produced NO could influence the TSH-stimulated thyroid cell proliferation. Two different approaches were used to evaluate the cell proliferation, the [^3H]thymidine

incorporation and the dye dilution assay using the intracellular fluorescent dye, CFSE.

FRTL-5 cells grown in culture medium with the addition of L-NMMA showed a significant increase of the TSH-stimulated [^3H]thymidine incorporation at 24 h (Fig. 7A). This effect persisted at 48 h (Fig. 7B). We also observed an increase in [^3H]thymidine incorporation by treatment of TSH-stimulated cells with L-NAME (Fig. 7C). The addition of L-NMMA or L-NAME alone did not modify [^3H]thymidine incorporation compared with the basal values (Fig. 7A).

The flow cytometric analysis of CFSE-stained cells has been widely used for the study of the cell proliferation in several cell types and brings about the advantage of identifying the number of cell divisions and the number of cells that undergo cell division in a given cell population (Lyons 2000, Wang *et al.* 2005). We observed an increase in the number of divisions and in the proportion of cells in each phase of the cell cycle in TSH-stimulated FRTL-5 cells cultured with the addition of L-NMMA when compared with cells in the presence of TSH alone (Fig. 8A and B). The addition of L-NMMA alone did not modify cell division in basal conditions (data not shown). These results are in concordance with those obtained in [^3H]thymidine incorporation assays. These observations seem to indicate that the endogenously produced NO is able to reduce the TSH-induced thyroid cell proliferation.

TSH stimulated the production of NO

From these results, a role for NO in the regulation of thyroid hormone biosynthesis was suggested. Thus, it was of interest to analyse whether the endogenous NO production was TSH-dependent. FRTL-5 cells were treated with different concentrations of TSH (100, 200 and 500 $\mu\text{U}/\text{ml}$) for different times (12, 24 and 48 h) and NO generation was estimated by L-[^{14}C]citrulline production after incubation with L-[^{14}C]arginine. As depicted in Fig. 9A, incubation of cells with TSH significantly increased the L-[^{14}C]citrulline generation. The

Figure 7 NOS inhibition increased TSH-stimulated [^3H]thymidine incorporation. (A) Cells were grown for 2 days to ~40% of confluence. Cells were shifted to basal medium for 4 days and then treated with TSH alone or in the presence of 0.1 mM L-NMMA for 24 h. Data are expressed as % above basal (taken as 0%; basal value = 9709 c.p.m./well). Data represent the mean $\pm$ S.E.M. of at least three independent experiments. (B) Cells grown as in A were treated with 200 $\mu\text{U}/\text{ml}$ TSH with or without 0.1 mM L-NMMA for 24 and 48 h. Data are expressed as % above basal (taken as 0%, basal value = 1444 c.p.m./well). Results are given as mean $\pm$ S.D. of a representative experiment. Similar results were obtained in two additional experiments. (C) Cells grown as above were treated with 200 $\mu\text{U}/\text{ml}$ TSH alone or in the presence of 1 mM L-NAME for 24 h. Data are expressed as % above basal (taken as 0%). Results are given as mean $\pm$ S.D. of a representative experiment. Similar results were obtained in two additional experiments. * $P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ vs TSH alone; $^{\S}P < 0.05$, $^{\parallel}P < 0.01$, $^{\P}P < 0.001$ vs basal (without TSH); Student–Newman–Keuls multiple comparisons test.

Figure 8 L-NMMA increased TSH-stimulated thyroid cell proliferation measured by CFSE labelling. FRTL-5 cells were labelled with 7.5 µM CFSE, maintained for 3 additional days in basal medium and then treated with 200 µIU/ml TSH alone or in the presence of 0.1 mM L-NMMA. After 3 days of culture, cells were harvested and analysed by flow cytometry. (A) Histograms of CFSE fluorescence on the horizontal axis (logarithmic scale). Markers above the peaks were used to enumerate events of each division cycle in cells cultured with L-NMMA. The stainings of basal cells (filled grey histograms), cells cultured with 200 µIU/ml TSH alone (open light line histograms) or in the presence of 0.1 mM L-NMMA (open black line histograms) are shown for comparison. (B) The graphic shows the mean fluorescence intensity values $\pm$ s.d. from the same data points of a representative experiment. Similar results were obtained in two additional experiments.

stimulatory effect of TSH on NO production was registered with 200 and 500 µIU/ml at 24 h (Fig. 9A). The maximal increase was observed at 24 h (Fig. 9B). The NOS inhibitor L-NMMA was used to test specificity for the NOS-dependence of the L-citrulline increment. Pre-incubation with L-NMMA for 15 min abolished the TSH-induced NO production (Fig. 9A and B). Incubation with L-NMMA alone did not modify L-citrulline levels (Fig. 9A). These results indicated that the NOS-mediated production of NO in the thyrocyte could be, at least in part, under TSH regulation.

Discussion

This study provides evidence that the inhibition of NOS activity is associated with an increase of TSH-stimulated iodide uptake in the thyroid cell, suggesting an inhibitory action of the endogenously produced NO on this essential functional step of thyroid hormone biosynthesis. The increase of NIS mRNA level that we observed under NOS inhibition seems to indicate that the NO-induced reduction of iodide uptake could be related to a diminished NIS expression. In coincidence, here, we demonstrated that the TG mRNA is increased when NOS activity is blocked, which supports a negative action of NO on the TG gene expression. Our observations reveal the ability of the endogenously produced NO to inhibit differentiated functions of thyroid cells. These findings strongly agree with the proposed mediation of NO in the cytokine-induced reduction of the TSH-stimulated TPO and thyroid oxidase (ThOX) expression observed in human thyrocytes (Gerard *et al.* 2006).

The action of NO is controversial and complex because this active molecule exhibits dual effects depending on its concentration and the molecular environment. As a consequence, NO can act as a toxic or a protective agent on different cell types (Albrecht *et al.* 2003, Kroncke 2003). Previous studies have reported an inhibitory effect of NO donors on differentiation markers in thyroid cells from several species (Millatt *et al.* 1993, Kasai *et al.* 1995, Bocanera *et al.* 1997, Costamagna *et al.* 1998, Lucero *et al.* 2005). Thus, these findings evidenced that the exogenously provided NO is able to produce an inhibitory action on the thyroid cell function. Coincidentally, the present results support the hypothesis that the thyrocyte-produced NO could participate in a negative regulation of the thyroid hormone biosynthesis. Therefore, similar effects appear to be exerted by the endogenously produced and the exogenously supplied NO in the thyroid cell.

It has been reported that the activation of phosphoinositol/calcium cascade increased the cGMP level in dog thyroid slices (Van Sande *et al.* 1975, Esteves *et al.* 1992). This calcium-mediated cGMP production was decreased by NOS inhibition, supporting an endogenous NO-dependent cGMP accumulation (Esteves *et al.* 1992). The observations here obtained under blockage of cGK support the view that this kinase could be involved in the endogenous NO-mediated inhibition of iodide uptake. In accordance, a possible role of the cGMP/cGK pathway in the inhibitory effect of NO donors on iodide uptake has been proposed previously (Bocanera *et al.* 1997).

The increase in the functional activity of NIS and TG promoters when NOS activity is blocked indicates that NO could inhibit the TSH-induced thyroid-specific gene expression at the transcriptional level. It is well known that

Figure 9 TSH increased the production of NO in the thyroid cells. (A) Basal FRTL-5 cells were incubated with TSH (100–500 µU/ml) for 24 h. (B) Basal cells were treated with 200 µU/ml TSH for 12, 24 and 48 h. To control the specificity of the response, cells were pre-incubated for 15 min with 0.1 mM L-NMMA. Data are expressed as % above basal (taken as 0%; basal value = 377 c.p.m./µgDNA). Results are given as mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ vs basal (without TSH, increase); † $P < 0.05$ vs TSH alone (decrease); Student–Newman–Keuls multiple comparisons test.

the NIS and TG gene expressions are regulated by a combination of ubiquitous and thyroid-specific factors, such as thyroid transcription factor-1 (TTF-1), thyroid transcription factor-2 (TTF-2) and paired box domain transcription factor 8 (Pax 8). Several post-translational mechanisms, such as redox regulation and phosphorylation, have been demonstrated to control the activity of these factors (Kambe *et al.* 1996, Damante *et al.* 2001). Evidence is accumulating that NO preferentially modifies the activity or level of redox-sensitive transcription factors (Kroncke 2003). Then, it could be speculated that NO could partially act by modifying the activity or expression of thyroid-specific transcription factors. In favour of this hypothesis, our group recently demonstrated a NO-mediated reduction of TTF-2 mRNA expression in FRTL-5 thyroid cells (Lucero *et al.* 2005).

We observed an increase in the TSH-stimulated proliferation of FRTL-5 thyroid cells after disruption of NO production indicating an inhibitory effect of endogenous NO on cell proliferation. Controversial effects of NO on cell proliferation have been reported. In agreement with our results, the blockade of NOS activity by NOS inhibitors

(Lopez-Farre *et al.* 1997) or the ablation of NOS expression by antisense technology (Cartwright *et al.* 2000) increased endothelial cell proliferation. Contrarily, it was observed that the NO donor SNP stimulated endothelial cell proliferation (Ziche *et al.* 1994). Our findings show a novel property of endogenous NO to negatively regulate the TSH-induced thyroid cell proliferation.

The present study demonstrated for the first time a TSH-dependent production of NO in the thyroid cell. The fact that NOS inhibitors abolished the TSH-induced NO production indicates the mediation of NOS. Interestingly, findings in some pathological thyroid tissues appear to indicate a TSH-dependent NOS expression. Thus, it has been reported that NOS III expression was augmented in hyperthyroid tissues bearing a high activity of TSH receptor. It was observed that NOS III immunoreactivity was progressively increased at the apical cell membrane of tissues from Graves' disease, toxic adenomas and thyrocytes from a patient with an activating mutation of the TSH receptor, whereas it was barely detectable in hypothyroid tissues (Colin *et al.* 1997). Surprisingly, TSH increased NO production in human aortic endothelial cells (Donnini *et al.* 2003).

It has been demonstrated that several mechanisms are involved in the autoregulation of thyroid function. Thus, organic iodocompounds and TG have been reported to negatively autoregulate TSH-induced differentiation parameters in the thyroid cell (Pisarev 1985, Dunn & Dunn 2001, Eggo *et al.* 2003). From the present results, the endogenous NO production induced by TSH could be proposed as a possible inhibitor in the regulation of the thyroid function. In accordance, it has been reported a role of NO in modulating the function of other endocrine cells. Thus, endogenous NO inhibits the ACTH-stimulated aldosterone production in glomerulosa cells (Sainz *et al.* 2004).

In summary, the present findings provide evidence that the endogenous NO could act as a novel negative signal on the TSH-induced specific gene expression and proliferation in the thyroid cell. Since the thyroidal NO production seems to be TSH-dependent, it could be proposed to have a possible role in the regulation of thyroid cell function.

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