Expression of TPO and ThOXs in human thyrocytes is downregulated by IL-1α/IFN-γ, an effect partially mediated by nitric oxide

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Gérard, Anne-Catherine, Marie Boucquey, Marie-France van den Hove, and Ides M. Colin. Expression of TPO and ThOXs in human thyrocytes is downregulated by IL-1α/IFN-γ, an effect partially mediated by nitric oxide. Am J Physiol Endocrinol Metab 291: E242–E253, 2006. First published February 14, 2006; doi:10.1152/ajpendo.00439.2005.—Morphological and functional alterations in Hashimoto’s thyroiditis (HT) are predominantly mediated by Th1 cytokines through apoptotic cell death. This ultimate step could be preceded by functional injuries in thyroid hormone synthesis. The action of two Th1 cytokines (IL-1α/IFN-γ) on thyroperoxidase (TPO) and thyroid oxidase (ThOXs) expression was tested in human thyrocytes isolated from normal tissues, Graves’ disease (GD) tissues, and autonomous toxic nodules. There was no evidence of cell death. Nitric oxide (NO) release was induced by cytokines but was absent when Nω-nitro-L-arginine methyl ester (L-NAME) was coincubated. When thyrotropin (TSH)-incubated normal and GD thyrocytes were treated with IL-1α/IFN-γ, TPO and TSH protein and mRNA expression dropped, a decrease partially prevented by L-NAME, suggesting that NO acts as a mediator of Th1 effects. In thyrocytes from autonomous toxic nodules, the high level of TPO and ThOXs protein expression was not influenced by TSH or by cytokines, a finding partially reproduced when normal thyrocytes were treated with increasing concentrations of TSH. In conclusion, incubation of normal or GD thyrocytes with Th1 cytokines induces a significant reduction in TSH-increased expression of both TPO and ThOXs, an effect partially mediated by NO. The thyroid cell function can therefore be severely affected in HT, even when cells remain viable. In autonomous toxic nodules, cells become partially insensitive to exogenous Th1 cytokines.

Th1 cytokines; Hashimoto’s thyroiditis; thyroid oxidase; thyroperoxidase; interleukin-1α; interferon-γ

HASHIMOTO’S THYROIDITIS (HT) and Graves’ disease (GD) are the two most common organ-specific autoimmune diseases. Although located at each extreme of the spectrum of autoimmune thyroid diseases, they are pathophysiologically closely related. Indeed, they are both characterized by the infiltration of the parenchyma by inflammatory cells, including B and T cells, as well as macrophages. However, the immune response is mostly cell mediated in HT, in contrast with GD, where humoral immunity is predominant (29, 38). The classical outcome of HT is the gradual loss of thyroid functions leading to permanent hypothyroidism. Nevertheless, the daily medical practice clearly shows that the initial HT clinical presentation can be highly pleomorphic, with some patients rapidly developing glandular atrophy along with severe hypothyroidism, although others remain euthyroid or subclinically hypothyroid for a long period of time (13, 21, 39). In addition, some patients can sometimes escape from hypothyroidism and recover to an euthyroid function, suggesting the possible reversibility of the immunological injury. This could be due to transient alterations in the thyroid function rather than irreversible apoptotic cell death (22, 23). One may consider the involvement of Th1 cytokines, as already suggested (2). Th1 cytokines have been shown to be prominent in HT (8) and to have profound effects on the expression of various differentiation proteins, such as thyroglobulin (Tg), thyroperoxidase (TPO), and Na+/I− symporter (NIS) (6, 7, 30, 41). Thus, among various mechanisms, hypothyroidism in HT could also result from Th1 cytokine-induced failure in hormonogenesis.

The way Th1 cytokines are interacting with thyrocytes is complex. Previous papers (19, 25, 40) reported that some cytokine-induced effects in the thyroid gland could be mediated by nitric oxide (NO). The three NO synthase (NOS) isoforms are present and functionally active in the thyroid gland (9, 10). In addition, Th1 cytokines (IL-1α, IFNγ) were found to activate NOSII and induce NO release from human thyroid cells, thereby mediating their cytotoxic effects (40). Although NO does not mediate the effects of IL-1β on Tg and cyclic AMP (cAMP) production (31), we postulate here that Th1 cytokines could interfere with other thyroid cell functions, such as iodine organization and TPO activity, which are both known to be directly influenced by NO donors and guanosine 3’,5’-cyclic monophosphate (4, 11). To reproduce a simplified Hashimoto’s immunological milieu, we first tested the consequences of treating human thyroid cells in primary cultures with IL-1α together with IFN-γ (IL-1α/IFN-γ) on the expression of TPO and thyroid oxidases (ThOXs), two enzymatic systems required for thyroglobulin iodination/coupling and H2O2 production, respectively. To analyze the involvement of NO as mediator of Th1 cytokine actions, human thyrocytes were incubated with IL-1α/IFN-γ in the presence of 1-nitroarginine-methyl ester (l-NAME), a NOS inhibitor. We performed identical experiments in FRTL-5 cells, a rat thyroid cell line unable to release NO in response to Th1 cytokines (35). Finally, we determined whether results obtained in normal tissues were reproducible in fetal thyrocytes, in cells without previous exposure to immunological modulators, and in thyrocytes isolated from two types of functionally hyperactive adult tissues, an autoimmune disease (GD) and a nonautoimmune disease (autonomous toxic nodules).
METHODS

Human thyroid tissues. Five normal tissues (2 bordering on toxic nodules and 3 from multinodular goiters), two toxic nodules, and two thyroids from GD patients were obtained at surgery after patients gave their informed consent. The thyroid from one fetus was obtained after abortion for spina bifida at 22 wk after parents gave their informed consent. The mother was free of thyroid autoimmune disease, ruling out the likelihood of maternal antibody-induced reaction against fetal epitopes. A small fragment of each thyroid was rapidly frozen in liquid nitrogen for immunohistochemistry. The remaining tissue was used to isolate thyrocytes for cell culture.

Cell cultures. Human thyrocytes were isolated according to Nilsson et al. (27) and suspended in modified Earle’s medium (MEM) without phenol red containing 5% newborn calf serum (NCS), penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (2.5 μg/ml; BRL-GIBCO, Paisley, Strathclyde, UK). They were plated in six-well plates (40–50 μg DNA/well) or in 24-well plates (fetal cells, 8 μg DNA/well; VWR International, Leuven, Belgium) and cultured in a humidified atmosphere (5% CO2) without or with 1 μU/ml thyrotropin (TSH; Sigma, Bornem, Belgium) for 1 wk. Cells cultured without TSH were used as control. Culture medium was changed every 2 or 3 days. After 1 wk with TSH, cells were incubated for 3 additional days with recombinant human IL-1α (2 ng/ml; R&D Systems, Abingdon, UK) and recombinant human IFN-γ (100 U/ml, R&D Systems) in the same medium but containing 0.5% NCS. To investigate the role of NO, l-NAME (2.5 mM) was added with the cytokines.

FRTL-5 cells were a gift from Dr. P. Kopp (Northwestern University, Chicago, IL). They were grown to 80–90% confluence in Coon’s modified Ham’s F12 medium (GIBCO) supplemented with 5% NCS, 5 mU/ml TSH, 10 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml glycyrl-histidyl-l-lysine acetate, and 3.2
ng/ml hydrocortisone (6H medium; all reagents from Sigma). Cells were then incubated with 1 μg/ml insulin, without or with 1 mU/ml TSH for 3 days, and with recombinant rat IL-1α (2 ng/ml; Chemicon International, Temecula, CA) and IFN-γ (100 U/ml; Chemicon International) with TSH, for 3 additional days, in combination or not with l-NAME, in the same medium containing 0.5% NCS. Each experiment was repeated at least twice, except for fetal cells.

For each experimental condition, six wells were used for nitrite and viability assays. Thereafter, three wells were allocated for Western blotting and three others for reverse transcription-polymerase chain reaction (RT-PCR).

In an additional experiment, repeated twice, normal human thyrocytes were cultured with 1, 10, 20, or 50 mU/ml TSH for 10 days. Recombinant human IL-1α (2 ng/ml) and IFN-γ (100 U/ml) were added to the culture medium for the last 3 days.

**Nitrite assay.** Nitrite accumulation in the medium was measured by the Griess reaction using a commercially available kit (Promega, Madison, WI). Absorbance at 550 nm was measured with a Packard SpectraCount (Packard Instruments, Downer Grove, IL).

**Viability assay.** Cell viability was assessed using alamar blue assay (Biosource International, Camarillo, CA), which is based on the detection of the metabolic activity of cells that incorporate a fluorometric indicator. Alamar blue that has little intrinsic fluorescence is reduced in viable cells and thereby becomes highly fluorescent. Fluorescence level is therefore directly proportional to the number of living cells (26). Ten percent alamar blue in fresh culture medium was added to each culture well, and fluorescence was measured after 4 h in a 96-well plate using a Packard FluoroCount at an excitation wavelength of 330 nm and an emission wavelength of 460 nm.

**Western blotting.** Thyrocytes from three individual wells were suspended in Laemmli buffer containing a protease inhibitor cocktail (Sigma) and sonicated for 30 s. Protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL). Proteins (30 μg) were heated at 95°C for 5 min in the loading buffer [50 mM Tris·HCl, pH 6.8, with 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol], separated by 8% SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Roosendaal, The Netherlands). After being stained with Ponceau Red (Sigma) to check for protein transfer efficacy and loading regularity, destained membranes were blocked for 1 h at room temperature (rt) with 5% nonfat dry milk in PBS (pH 7.4) and 0.1% Tween and incubated overnight at 4°C with primary antibodies against ThOXs (polyclonal antibody directed against ThOXs1 and ThOXs2; gift from F. Miot, IRIBHN, Brussels, Belgium) or TPO (monoclonal antibody mAb47; gift from J. Ruf, Université de la Méditerranée, Marseille, France), both at a 1/4,000 dilution.
dilution in PBS-Tween/1% fat dry milk. Membranes were washed, incubated for 1 h at rt with peroxidase-labeled secondary antibody anti-rabbit (ThOXs, 1/20,000) or anti-mouse (TPO, 1/10,000; Amersham), washed again, and visualized with enhanced chemiluminescence (SuperSignal West Pico; Pierce) on CL-Xposure films (Pierce). In FRTL-5 cells, TPO expression was not analyzed because of the lack of a specific antibody against rat TPO.

**RT-PCR.** Thyrocytes from three individual wells were suspended in TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany), and total RNA was purified according to the manufacturer’s protocol. Reverse transcription was performed using a Moloney murine leukemia virus RT (M-MLV-RT; Invitrogen, Merelbeke, Belgium) on 2 μg of total RNA (or 5 μl of total RNA solution if RNA concentration was too low) in a reaction volume of 20 μl. One-fortieth of the cDNA product was used for PCR amplification using TaKaRa Taq DNA polymerase (Takara Bio, Shiga, Japan). For specific amplification of human ThOXs-1 and -2 cDNA, sense primer was 5'-GTGGCTGGCTGACATCAT-3' and anti-sense primer 5'-CCAGAGCTTGAAGAAACCTCAA-3'; for human TPO, sense primer was 5'-CACGATGCAAGAAACCTCAA-3' and anti-sense primer 5'-GAGGGAGCCATGAAGACAA-3'. RNA integrity and reverse transcription efficiency was checked by amplification of β-actin cDNA using 5'-CATCCTGCTGGACATCAT-3' as sense primer and 5'-GAGGAGGAGCAATGATCTTGAT-3' as anti-sense primer. Amplifications were performed with denaturation at 94°C for 3 min followed by cycles of 1 min at 94°C, 2 min at specific melting temperature, elongation step at 72°C for 1 min, and a final extension step of 10 min at 72°C. The number of cycles was selected after the linearity of the amplification reaction was verified. Nineteen cycles were used for β-actin, 23 for TPO, and 25 for ThOXs. Specific melting temperatures were 62°C for β-actin, 60°C for TPO, and 56°C for ThOXs. PCR products were separated by agarose gel (1 or 1.5%) electrophoresis. Control reactions using water were used to rule out the possibility of a genomic DNA contamination.

**Immunofluorescence.** Thyrocytes from normal tissues were cultured in MEM with 1 mU/ml TSH on glass coverslides precoated with type I collagen (Roche Diagnostics). Cells cultured without TSH were used as control. After 1 wk, IL-1α/IFN-γ was added or not added for 3 days. Thyrocytes were fixed for 20 min in 4% paraformaldehyde, rinsed once with PBS, permeabilized for 15 min in a PBS-Triton 1% solution at rt, and washed with PBS supplemented with 1% bovine serum albumin (PBS-BSA). Cells were then incubated either overnight with ThOXs primary antibody (1/50) or for 1 h with TPO antibody (1/500), both at rt. After being washed in PBS, fluorescein isothiocyanate-conjugated secondary antibodies were added for 1 h at rt at a dilution of 1/40 (anti-rabbit for ThOXs and anti-mouse for TPO; DAKO, Carpinteria, CA). Coverslides were mounted in fluorescent mounting medium (DAKO) for microscopic observation. Negative controls included omission of the primary antibodies.

Fluorescence was observed under a Leitz fluorescence microscope (Leitz, Troisdorf, Germany), and pictures were taken with a Nikon Coolpix 4500 digital camera (Nikon, Tokyo, Japan).

**Immunohistochemistry.** Immunostaining was performed at rt as previously described (17). Five-micrometer cryostat sections were...
plunged into acetone, rinsed with PBS-BSA, and incubated for 30 min in PBS-BSA containing normal goat serum (Vector Labs, Burlingame, CA) at a dilution of 1/50. The first antibody was then applied (ThOXs 1/75 or TPO 1/3,000) for 1 h. After two washes in PBS-BSA, the antibody binding was detected with a second antibody conjugated to a peroxidase-labeled polymer (Envision Detection; DAKO) for 30 min at rt. After washing, peroxidase activity was revealed with the AEC substrate (3-amino-9-ethylcarbazole; DAKO). Sections were counterstained with Mayer’s hematoxilin, rinsed, and mounted in Faramount (DAKO). Several negative controls were performed, including absence of the first antibody or substitution of the first antibody by preimmune serum.

**Data analysis and statistics.** Data are means ± SE. Six individual culture wells were tested for nitrite and viability assays. Statistical analyses were performed using ANOVA followed by Tukey-Kramer multiple comparisons test (GraphPad InStat, San Diego, CA), and P < 0.05 was considered as significant.

Western blots were scanned and quantified by densitometry using the NIH Scion Image Analysis Software (National Institutes of Health, Bethesda, MD). For cells from normal tissues, values expressed as percentage of TSH-treated cells were calculated in 3, 4, or 5 different tissues and expressed as means ± SE. For cells from toxic nodules, GD, and TSH experiment, values are expressed as means ± SE of one representative experiment performed in triplicate (n = 3 wells).

**Fig. 4.** TPO and ThOXs protein and mRNA expression in thyrocytes from normal human thyroids. A and C: Western blot analysis of TPO (A) and ThOXs (C) protein expression. Densitometric values are expressed as %TSH-treated cells. They were obtained from 5 experiments [TSH-incubated and IL-1α/IFN-γ-treated cells, (n = 5)] or from 4 different experiments [cells cultured without TSH and cells coincubated with l-NAME (n = 4)], except for ThOXs without TSH, where only 3 experiments were considered (n = 3). Th1 cytokine-induced decrease of TPO and ThOXs protein expression was in part prevented by l-NAME. B and D: RT-PCR detection of TPO (B) and ThOXs (D) mRNAs. Densitometric values, adjusted to β-actin signal, are expressed as %TSH-treated cells. They are shown for 3 different experiments (n = 3), except for l-NAME, where only 2 experiments were considered (n = 2). Th1 cytokine-induced decrease of TPO and ThOXs mRNA expression were not prevented by l-NAME. Each dot is the mean of values obtained in 3 wells. *P < 0.05 vs. control cells; +P < 0.05 vs. TSH-treated cells; §P < 0.05 vs. TSH-treated cells incubated with IL-1α/IFN-γ from day 7 to day 10; ■ control cells; ▲ TSH-treated cells; ▼ TSH-treated cells incubated with IL-1α/IFN-γ; ● TSH-treated cells incubated with IL-1α/IFN-γ and l-NAME.
Results of ethidium bromide-stained agarose gels were quantified by densitometry using the Scion Image Analysis software. Digital imaging values were adjusted to β-actin expression. For normal tissue, individual ratios were calculated in 2 or 3 thyroids and expressed as percentage of TSH-treated cells. For toxic nodules, GD, and TSH experiment, values are expressed as means ± SE (expressed as ThOXs/β-actin or TPO/β-actin ratio) of one representative experiment performed in triplicate (n = 3 wells).

For normal tissues, statistical analysis was performed using paired t-test. For cells obtained from a toxic nodule, GD tissue, and cells treated with TSH, the unpaired t-test with Welch correction or the nonparametric Mann-Whitney test was used, with P < 0.05 considered as statistically significant.

RESULTS

**TPO and ThOXs are expressed at the apical pole of thyrocytes.** The immunohistochemical analysis prior to culture showed a positive signal for TPO and ThOXs in all tissues. Compared with the corresponding normal tissue (Fig. 1, A and B), protein expression was strongly enhanced in thyrocytes from an autonomous toxic nodule, especially for TPO (Fig. 1C), and in an apical position for ThOXs (Fig. 1D). In GD thyroids, TPO (Fig. 1E) and ThOXs (Fig. 1F) were located at the apical pole of thyrocytes, and their expression was less increased than in the toxic nodule. In the 22-wk-old fetal thyroid, TPO was restricted to the apical compartment of the cytoplasm (Fig. 1G), whereas ThOXs appeared as a sharp apical line (Fig. 1H). These results indicate that the expression of the two proteins of interest, before cell dissociation, is regulated as previously reported (14).

**Th1 cytokines induce NO production without affecting cell viability.** NO production was evaluated by measuring nitrite accumulation, the stable end product of NO generation, in culture medium. Under basal conditions, with or without TSH, nitrite levels were very low in media from human normal cells. In contrast, nitrite release was induced by 12-fold after 3 days with IL-1α/IFN-γ and completely blocked after coincubation with L-NAME (Fig. 2A), as previously reported (40). An identical observation was made in culture media of fetal thyrocytes (Fig. 2B), toxic nodule (Fig. 2C), and GD cells (Fig. 2D). In contrast, FRTL-5 cells failed to release NO in the culture medium (data not shown), which is in accordance with a previous publication (35).

In all experiments, neither cell viability nor cell morphology was affected by the IL-1α/IFN-γ treatment, indicating that the observed Th1-induced effects did not result from cell death (data not shown).

**Expression of TPO and ThOXs in normal human thyrocytes is downregulated by IL-1α/IFN-γ at protein and mRNA levels.** In normal human cells cultured for 1 wk without TSH on glass coverslides, TPO protein expression was not detectable by
immunofluorescence, whereas ThOXs expression was low (Fig. 3, A and B). After 1 wk with TSH, a strong signal was observed for both proteins (Fig. 3, C and D). In contrast, in TSH-treated cells supplemented with IL-1α/IFN-γ for 3 days, the signal nearly disappeared for TPO (Fig. 3E) and was strongly reduced for ThOXs (Fig. 3F). Western blot analysis showed that the TPO band at 110 kDa was faint after 7 days of culture without TSH and 4-fold stronger when TSH was present (P < 0.05). IL-1α/IFN-γ treatment significantly decreased by 59% (P < 0.05) the TSH-induced expression of TPO (Fig. 4A). ThOXs protein expression (179 kDa), although slightly but not significantly affected by TSH, was also significantly decreased after IL-1α/IFN-γ treatment (63% decrease (P < 0.05; Fig. 4C). RT-PCR analysis showed that TPO and ThOXs mRNA expression was low without TSH and upregulated by TSH (4-fold and 2-fold increase for TPO and ThOXs, respectively, P < 0.05). In thyrocytes incubated with IL-1α/IFN-γ, the mRNA contents returned to values measured in cells cultured without TSH (P < 0.05; Fig. 4, B and D).

A comparable variation of both TPO (Fig. 5, A and B) and ThOXs (Fig. 5, C and D) protein and mRNA expression was observed in one culture of fetal thyrocytes. Results should here be viewed as qualitative instead of quantitative. Indeed, the scarcity of cells did not allow for systematically performing this specific experiment twice or in triplicates, especially for the Western blot analysis and for the group without TSH in RT-PCR analyses.

In rat FRTL-5 cells cultured without TSH, a ThOXs band of 179 kDa was observed, but, in contrast with results obtained in human cells, its intensity was faint. The twofold increase in

![Fig. 6. TPO and ThOXs protein and mRNA expression in thyrocytes from a GD goiter. A and C: Western blot analysis of TPO (A) and ThOXs (C) protein expression. Densitometric values are expressed as means ± SE of 1 representative experiment performed in triplicate (n = 3). Th1 cytokine-induced decrease of TPO and ThOXs protein expression was prevented by L-NAME. B and D: RT-PCR detection of TPO (B) and ThOXs (D) mRNAs. Densitometric values, adjusted to β-actin signal, are expressed as means ± SE of 1 representative experiment performed in triplicate (n = 3). Th1 cytokine-induced decrease of TPO and ThOXs mRNA expression was prevented by L-NAME. *P < 0.05 vs. control cells; **P < 0.05 vs. TSH-treated cells; ○P = 0.05; ◦P < 0.05 vs. TSH-treated cells incubated with IL-1α/IFN-γ from day 7 to day 10; NS, nonsignificant.](http://ajpendo.physiology.org/)
ThOXs protein expression induced by TSH ($P < 0.05$) was reduced by half ($P < 0.05$) after IL-1α/IFN-γ addition (data not shown).

The effect of Th1 cytokines is partially mediated by NO in normal human thyrocytes. In Th1-treated normal thyrocytes, l-NAME cotreatment partially prevented cytokine-induced decrease in TPO protein expression ($P < 0.05$), whereas l-NAME only slightly protected ThOXs expression in two of four cases (Fig. 4, A and C). In contrast, l-NAME did not modify the cytokine-induced reduction in TPO and ThOXs mRNA contents (Fig. 4, B and D).

In fetal cells, l-NAME cotreatment also prevented the decrease in TPO (Fig. 5A) and ThOXs protein expression (Fig. 5C) without affecting TPO (Fig. 5B) and ThOXs (Fig. 5D) mRNA expression.

Fig. 7. TPO and ThOXs protein and mRNA expression in thyrocytes from an autonomous toxic nodule. A and C: Western blot analysis of TPO (A) and ThOXs (C) protein expression. Densitometric values are expressed as means ± SE of 1 representative experiment performed in triplicate ($n = 3$). TPO and ThOXs protein expression was influenced neither by TSH nor by Th1 cytokines. B and D: RT-PCR detection of TPO (B) and ThOXs (D) mRNAs. Densitometric values, adjusted to β-actin signal, are expressed as means ± SE of 1 representative experiment performed in triplicate ($n = 3$). Th1 cytokine-induced decrease of TPO mRNA expression was not prevented by l-NAME. ThOXs mRNA expression was influenced neither by TSH nor by Th1 cytokines. *$P < 0.05$ vs. control cells; †$P < 0.05$ vs. TSH-treated cells.

The Th1 cytokine-induced decrease in TPO and ThOXs expression is mediated by NO in thyrocytes from patients with GD. In thyrocytes isolated from two autoimmune GD goiters, TPO and ThOXs protein (Fig. 6, A and C) and mRNA (Fig. 6, B and D) expression were quite low in cells cultured without TSH and largely increased by TSH. Th1 cytokines induced a marked decrease in TPO (43% decrease, $P < 0.05$; Fig. 6A) and ThOXs (53% decrease, $P < 0.05$; Fig. 6C) protein expression. Th1 cytokine-induced effects were prevented by l-NAME (compared with IL-1α/IFN-γ-treated cells, $P \leq 0.05$). TPO and ThOXs mRNA expression followed the same pattern of variation as the respective proteins. Thus TSH-induced upregulation of TPO (Fig. 6B) and ThOXs (Fig. 6D) mRNAs expression was inhibited by IL-1α/IFN-γ, an effect prevented by l-NAME ($P \leq 0.05$).
Th1 cytokines do not exert significant effects on TPO and ThOXs expression in thyrocytes isolated from autonomous toxic nodules. In thyrocytes isolated from two different autonomous toxic nodules, the expression of TPO (Fig. 7A) and ThOXs (Fig. 7C) proteins was insensitive to both TSH and cytokines without or with L-NAME. However, TSH induced a significant upregulation of TPO mRNA expression (2.5-fold, P < 0.05) that was abrogated by IL-1α/IFN-γ, without any effect of L-NAME (Fig. 7B). In contrast, ThOXs mRNA expression was influenced by neither TSH nor Th1 cytokines (Fig. 7D).

Normal thyrocytes treated with high doses of TSH tend to behave as cells from toxic nodule. To test the role of the constitutively hyperstimulated status of cells from toxic nodules in their resistance to Th1 cytokines, we treated normal thyrocytes with increasing concentrations of TSH for 7 days, without or with cytokines for 3 additional days. Independent of TSH concentration, cytokine treatment induced NO production (Fig. 8). Of note, this effect was inversely proportional to the TSH concentration. Western blot analysis showed that TPO expression was TSH dependent. Compared with control, a maximal induction was reached at a TSH concentration of 10 mU/ml and did not further increase at higher doses of TSH. Th1 cytokine-induced reduction in TPO protein expression was maintained up to TSH levels of 20 mU/ml but was less pronounced at a TSH concentration of 50 mU/ml (Fig. 9A). ThOXs protein expression was not influenced by TSH. Cytokines induced a reduction in its expression independently of the TSH concentration (Fig. 9C). RT-PCR analysis showed that TPO mRNA expression (Fig. 9B), similar to protein expression, was TSH dependent in contrast with ThOXs mRNA expression (Fig. 9D). TPO mRNA expression was clearly decreased by Th1 cytokines up to 20 mU/ml of TSH. Concerning ThOXs mRNA expression, we observed that Th1 cytokine-induced effects were significant up to TSH levels of 10 mU/ml.

**DISCUSSION**

The present study aimed to evaluate the impact of two Th1 cytokines (IL-1α/IFN-γ) on the expression of TPO and ThOXs, two important enzymes for thyroid hormone synthesis, and the eventual implication of NO as a mediator of their effects. We report that IL-1α in combination with IFN-γ downregulates TPO and ThOXs protein and mRNA expression in human normal adult and fetal thyrocytes. IL-1α and IFN-γ are among Th1 cytokines that are released in greatest amounts in HT, a disease that usually evolves towards hypothyroidism (34). Because TPO and ThOXs are two key enzymes for thyroid hormone synthesis, the observed downregulation of their expression upon cytokine treatment may partly account for the hormone deficit that characterizes the disease. The hypothyroid status could therefore result not only from cell destruction by T lymphocyte-induced cell apoptosis (22, 23) but also from thyroid hormone synthesis failure because of interferences in iodide transport and organification. IL-1- and IFN-induced disturbances in thyrocyte functions have already been widely reported in previous studies (1, 3, 31–33, 35). More recently, IFN-γ has been reported to affect the expression of the iodide transporter NIS in a transgenic model of targeted IFN-γ expression in the thyroid gland (7). In addition, Caraccio et al. (6) reported that IFN-α and -β inhibit TSH-stimulated expression of thyroglobulin, TPO, and NIS genes in human thyrocytes. When these results are merged with ours, it clearly appears that IL-1 and IFN broadly cause inhibition of thyroid cell functions, even in fetal cells. The present study reports for the first time the additional effect of Th1 cytokines on ThOXs expression.

Incubation of thyroid cells with IL-1α/IFN-γ did not influence cell morphology or viability in our model of human cells in primary culture or in FRTL-5 cells, demonstrating that the variations in TPO and ThOXs expression were not due to cell destruction. Previous studies (3, 5, 20, 24, 32) addressing IL-1-induced alterations in thyroid cell growth, morphology, and apoptosis showed variable results but were primarily generated with IL-1β. Hence, human thyrocytes incubated with IL-1β exhibited striking morphological changes, including cytoplasmic retraction or dissociation (3). Treatment of thyrocytes with IL-1β/IFN-γ made these cells more sensitive to Fas-activated apoptosis and also increased Fas antigen expression on thyrocytes, at least without TSH (5, 20, 24). All data reported so far suggest an induction of thyroid cell apoptosis by IL-1β. Therefore, the specific effects induced by different cytokines should be carefully distinguished. For example, growth of FRTL-5 cells is inhibited by IL-1β but not by IL-1α (32). It is plausible that, preceding death, immunological injuries could affect thyroid cell functions and that defects in hormone synthesis might appear before cell destruction or, at least, independently from it. Although data obtained from an in vitro model may not be directly extrapolated to a more complex in vivo setting, one could reasonably propose that some specific Th1 cytokine subgroups preferentially provoke a failure in thyroid hormone synthesis through the downregulation of TPO and ThOXs expression and activity, and/or of iodide uptake, instead of immediately inducing hypothyroidism by cell destruction. This could be an alternative mechanism to explain why some patients bearing a thyroid autoimmune disease cycle between clinical or subclinical hypothyroidism.
and euthyroidism, sometimes long before becoming permanently hypothyroid (39).

NO is involved in a large variety of events occurring in the thyroid gland. It acts as a regulator of the thyroid microvasculature (9, 10, 15–17, 37), intervenes in the maintenance of the thyroid economy, and influences thyroid epithelial cell function (4, 11). Because NO is also a well-established mediator of the IL-1 signaling pathway and is produced in thyrocytes in response to IL-1α/IFN-γ incubation (19, 31, 40), we used L-NAME, a NOS inhibitor, to assess whether NO acts as a second messenger of IL-1α/IFN-γ-induced effects on TPO and ThOXs expression. In thyrocytes isolated from normal human tissues, inhibition of NO production by L-NAME prevented, but only partially, the fall in TPO and ThOXs protein expression. These results clearly indicate that IL-1α/IFN-γ actions are in part mediated by NO. This finding is in accordance with our previous report (40) that showed that L-NAME blocks IL-1α/IFN-γ-induced LDH release, a marker of cell cytotoxicity, but fails to restore transepithelial resistance, indicating that NO acts as a mediator of cytokine-induced cytotoxicity but not of disruption of the thyroid epithelial barrier. Nearly a decade ago, Reimers et al. (35) observed that the inhibition of thyroid function by IL-1α/IFN-γ was independent of NO in FRTL-5 cells. We draw the same conclusion by showing that the alteration of ThOXs expression by IL-1α/IFN-γ is also independent of NO in FRTL-5 cells. This suggests that activated

Fig. 9. Effect of increasing TSH concentrations on TPO and ThOXs protein and mRNA expression. A and C: Western blot analysis of TPO (A) and ThOXs (C) protein expression. Densitometric values are expressed as means ± SE of 1 representative experiment performed in triplicate (n = 3). Th1 cytokine-induced decrease of TPO protein expression was maintained up to TSH levels of 20 mU/ml. Th1 cytokine-induced decrease of ThOXs protein expression was independent of the TSH concentration. B and D: RT-PCR detection of TPO (B) and ThOXs (D) mRNAs. Densitometric values, adjusted to β-actin signal, are expressed as means ± SE of 1 representative experiment performed in triplicate (n = 3). Th1 cytokine-induced decrease of TPO and ThOXs mRNA expression was significant up to TSH levels of 20 and 10 mU/ml, respectively. *P < 0.05 IL-1α (2 ng/ml)/IFN-γ (100 U/ml)-treated cells vs. cells treated with the equivalent TSH concentration without cytokines; ●P < 0.05 vs. cells incubated with TSH (1 mU/ml) and IL-1α (2 ng/ml)/IFN-γ (100 U/ml) from day 7 to day 10.
intracellular pathways may be quite different in rat and human cells, although resulting in the same effect. In addition, this illustrates an important issue confirming that data obtained from one cellular system should not systematically be extrapolated to another one. Our analysis of TPO and ThOXs mRNAs also showed that NO is not the sole messenger for all intracellular targets hit by IL-1α/IFN-γ. Thus the preservation of protein expression without significant change in mRNA levels in L-NAME-treated normal cells implies a role for NO at a translational and/or posttranslational level rather than at the transcriptional level. Indeed, in our model, NO exerts a role on TPO and ThOXs protein expression but has little impact on mRNA expression. One likely way NO controls TPO and ThOXs proteins could result from their S-nitrosylation that regulates ubiquitination-dependent protein degradation (18).

As already suggested by others, it appears that IL-1α/IFN-γ effects may involve interferences between cytokine- and TSH-activated pathways that would lead to the inactivation of the latter through impaired cAMP production (32, 33). In addition, the thyroid-specific transcription factor-1 has been identified as a potential cellular target for IFN-γ actions in FRTL-5 cells (28). More recently, chronic exposure to IFN-γ has been reported to impair cAMP-response-element- and activating protein-1-regulated gene expression in rat thyroid cells (36).

Together with our data, these results emphasize that the way cytokines affect thyroid cells can be highly diversified, with multiple different intracellular targets that may, in part, be specific for each cytokine. The analysis of cells obtained from autonomous toxic nodules gave somewhat different results. In these constitutively stimulated cells, cytokines had no effect on TPO and ThOXs protein expression, whereas NO was still released. The immunohistochemical study on whole tissue before cell culture clearly showed that TPO immunolabeling was stronger than in normal thyroid. In addition, TPO and ThOXs proteins were strongly expressed in cultured thyrocytes without TSH, indicating that the cells conserved some of their original characteristics, such as their autonomous properties, even after 10 days of culture. The lack of a cytokine effect in cells with an activated cAMP pathway suggests a partial “resistance” to cytokines. This phenomenon, which may be associated with the functional status of the cells, is replicated, at least in part, by treatment of normal cells with high TSH concentrations. Our results are in accordance with papers reporting opposing effects between TSH and cytokines (28, 32, 33, 36). Indeed, TSH has been reported to impair IFN-γ-induced Fas expression (20). Compatible with a similar mechanism, here we report that cytokine-induced alterations in TPO and ThOXs expression can be counterbalanced by constitutively active mutation of the TSH receptor or high concentrations of TSH. Nevertheless, cytokine-induced NO production attests that cytokines convey a signal into cells. However, it is most likely different from the effects in normal cells, or it may be altered because of the hyperactive status of the cells. Thus the cellular resistance to cytokine-induced effects could be linked to dominance of the cAMP signaling pathway over other signal-transducing components, as recently reported by Eslzinger et al. (12). Using cDNA arrays, these authors reported that several genes involved in the expression of signal transduction proteins are actually downregulated in autonomous nodules. Although the methods used in our paper do not allow verification of this hypothesis, it is possible that dominance of a cAMP-driven transduction pathway could account for the lack of Th1 cytokine-induced effects in autonomous toxic nodules.

The importance of knowing the cell origin before interpreting results is further reinforced by the analysis of thyrocytes obtained from GD goiter. Here, TPO and ThOXs protein expression was quite low in cells cultured without TSH but strongly enhanced by TSH, confirming that, in contrast with toxic nodule, thyrocytes from GD remain sensitive to TSH. IL-1α/IFN-γ effects were basically similar to those observed in normal tissues, with a significant fall in TPO and ThOXs expression that, in this case, was totally prevented by L-NAME.

In conclusion, our results show that treating normal human thyroid cells with IL-1α/IFN-γ does not induce cell death but greatly alters TPO and ThOXs protein expression, thereby likely affecting the ability of the thyrocytes to maintain an adequate hormone synthesis. Th1 cytokine-induced effects are mediated by NO, at least partially, only in human cells and at a posttranslational level. Our results do not preclude the obvious involvement of many other intracellular pathways in this paradigm.

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