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Am J Physiol Cell Physiol 291:327-336, 2006. First published Feb 22, 2006;
doi:10.1152/ajpcell.00316.2005

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Thyroid hormones increase inducible nitric oxide synthase gene expression downstream from PKC-ζ in murine tumor T lymphocytes

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Submitted 30 June 2005; accepted in final form 15 February 2006

Barreiro Arcos, María Laura, Gabriela Gorelik, Alicia Klecha, Ana María Genaro, and Graciela A. Cremaschi. Thyroid hormones increase inducible nitric oxide synthase gene expression downstream from PKC-ζ in murine tumor T lymphocytes. *Am J Physiol Cell Physiol* 291: C327–C336, 2006. First published February 22, 2006; doi:10.1152/ajpcell.00316.2005.—Regulation of cell proliferation by thyroid hormone (TH) has been demonstrated, but the effect of THs and the mechanisms involved in lymphocyte activity have not been elucidated. Differential expression of PKC isoenzymes and high nitric oxide synthase (NOS) activity have been described in tumor T lymphocytes. We have analyzed the direct actions of TH on normal T lymphocytes and BW5147 T lymphoma cells in relation to PKC and NOS activities. THs increased tumor and mitogen-induced normal T lymphocyte proliferation. PKC isoenzyme-selective blockers impaired these effects in both cell types, indicating the participation of Ca^{2+}-dependent and -independent isoenzymes in normal and tumor cells, respectively. TH actions were blunted by extra- and intracellular Ca^{2+} blockers only in normal T lymphocytes, whereas NOS blockers impaired TH-induced proliferation in T lymphoma cells. Incubation for 24 h with TH induced a rise in total and membrane-associated PKC activities in both cell types and led to a rapid and transient effect only in tumor cells. THs increased atypical PKC-ζ expression in BW5147 cells and classical PKC isoenzymes in mitogen-stimulated normal T cells. TH augmented NOS activity and inducible NOS protein and gene expression only in tumor cells. Blockade of PKC and the atypical PKC-ζ isoform inhibited TH-mediated stimulation of inducible NOS and cell proliferation. These results show, for the first time, that differential intracellular signals are involved in TH modulation of lymphocyte physiology and pathophysiology.

3,5,3′-*l*-triiodothyronine; thyroxine; T lymphoma; protein kinase C isoenzymes

THYROID HORMONES (THs) exert a broad range of effects on development, growth, and metabolism. Both 3,5,3′-*l*-triiodothyronine (T_3) and thyroxine (T_4) influence proliferation and differentiation in different cell types (1, 11, 14, 25), but their direct actions on lymphoid cells have not been elucidated. Reports analyzing in vitro actions of THs on mitogenic responses of lymphocytes have been few and controversial. Although all evidence indicates that THs can alter lymphocyte reactivity, the direction and magnitude of the alteration appear to depend on the TH encountered by the responding cells, its concentration, and the source (i.e., species) of the lymphoid cells (2, 19, 30). However, the physiological mechanisms underlying TH effects have not been studied.

A coherent body of evidence suggests that THs modulate multiple neoplasia-dependent mechanisms (17, 39). In lymphoid cells, actions of TH in vitro have demonstrated enhancement of murine lymphocytic leukemia cell growth (5), as well as induction of cell apoptosis in Jurkat human T lymphoma cells (27).

On the other hand, protein kinase C (PKC) is critical for T lymphocyte activation and proliferation (41), whereas nitric oxide (NO) synthase (NOS) may function as an activator and an inhibitor of T cell apoptosis. We recently studied enzymatic activities in normal and tumor T lymphocytes and found differential participation of PKC and NOS in cell proliferation (15). BW5147 lymphoma T cells overexpressing the PKC-ζ isoform display high inducible NOS (iNOS) activity related to cell growth that is almost undetectable in normal or mitogen-stimulated T lymphocytes (3, 15). Moreover, it has been reported that NOS activation depends on the atypical PKC-ζ isoform in these cells (15), suggesting that these signals might participate in the multistep process leading to malignant transformation in BW5147 cells.

On the basis of these findings, to further assess the direct actions of TH on the function of normal and tumor T lymphocytes, cell proliferation was evaluated in vitro in resting and mitogen-stimulated normal T lymphocytes and in the T lymphoma cell line BW5147. We found a dose-dependent increase in tumor- and mitogen-induced proliferation of T cells but no effect in resting T lymphocytes. Participation of PKC and NOS, together with the individual isoenzymes involved in TH actions, was analyzed for the first time. These results will contribute to our understanding of TH actions in T lymphocyte physiology and pathophysiology.

MATERIALS AND METHODS

Cell suspension and culture conditions. The murine tumor cell line BW5147 (a generous gift from Dr. A Schimpl, Institute für Virologie und Immunobiologie, Universität der Würzburg) is a T cell lymphoma $CD3^+$ that expresses T cell αβ-receptor, as shown routinely by flow cytometry with specific antibodies (15). The cells were cultured at an optimal concentration of $1–5 \times 10^5$ cells/ml in RPMI 1640 (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 2 mM glutamine (GIBCO BRL), and antibiotics (GIBCO BRL), with twice weekly splitting after they reached exponential growth. Synchronized BW5147 cells, which had been kept in FBS-deprived medium for 24 h, were also used (10).

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Aseptically prepared lymphoid cell suspensions were obtained from lymph nodes of 6- to 90-day-old female BALB/c inbred mice (Instituto Nacional de Tecnología Agropecuaria). The animals were housed in standard conditions of light and temperature, and their care conformed to the principles and guidelines of the National Research Council (1996) and approved by the Animal Care and Use Committee of the Centro de Estudio Farmacológicos y Botánicos—CONICET–School of Medicine. T cells were purified as described previously (7). Cells (1×10^6 cells/ml) were cultured in the same medium as tumor cells, alone or in the presence of concanavalin A (ConA, 2 μg/ml; Sigma Chemical), as previously described (7, 15).

The cells, at a final volume of 0.2 ml, were moved to 96-well flat-bottom microtiter plates (Nunc) for microcultures or kept in T-25 or T-75 culture flasks (Corning) for macrocultures. The cells were cultured for different times, and TH, T_3 or T_4 (Sigma Chemical), was added at the beginning of the culture.

To avoid the influence of the hormones in FBS, cells were also cultured in a serum-free medium (CCM1, HyQ, HyClone). T lymphoma cells were adapted to the serum-free environment by the immersion method; for mitogen stimulation of normal T lymphocytes, cells were purified and settled in this medium and cultured as indicated for the FBS-supplemented medium.

Levels of T_3 and T_4 in FBS were determined by a commercial RIA kit (Diagnostic Products) and found to be 117.3 ± 12.4 ng/dl and 8.0 ± 1.0 μg/dl, respectively.

Proliferation assays. Proliferation was evaluated on microcultures. As control of TH effects, proliferation was also evaluated on 0.2-ml aliquots of macrocultures established for enzymatic assays. Cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR, 20 Ci/mmol; NEN) for the last 6 h of incubation, as described elsewhere (7, 15). Results are expressed as disintegrations per minute (dpm) in experimental cultures, with subtraction of the control values obtained on BW5147 synchronized cells. For ConA-stimulated T lymphocytes, cultures were pulsed with [^{3}H]TdR as indicated for BW5147, and cultures of unstimulated cells were used as controls.

Carboxyfluorescein diacetate succinimidyl ester labeling. BW5147 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using a cell tracer kit (Vybrant CFDA SE, Molecular Probes) according to the manufacturer's instructions for cells in suspension. Briefly, 1×10^7 cells were washed twice with PBS and then resuspended gently in prewarmed PBS containing the probe (5 μM CFSE). The cells were incubated for 15 min at 37°C, repelleted by centrifugation, and resuspended in fresh prewarmed medium. The cells were incubated for another 30 min to ensure complete modification of the probe and then washed again and cultured in RPMI 1640 without FBS and in the presence or absence of 10^{-9} M T_3. At the indicated times, $\sim 1 \times 10^6$ cells were obtained and fixed for 15 min at room temperature using 3.7% formaldehyde. After fixation, the cells were rinsed in PBS and analyzed by flow cytometry. Results of events vs. fluorescence intensity were obtained for each sample with the WinMDI 2.8 program, and the number of cell divisions was calculated as described by Cooperman et al. (9).

PKC assay. Synchronized BW5147 cells (0.5×10^7 cells/sample) were recultured in the absence or presence of T_3 or T_4 and immediately frozen in liquid N_2. Normal or ConA-stimulated T cells (1×10^7 cells/sample) were incubated alone or in the presence of TH and processed as tumor cells. PKC was purified from total cell extracts or from subcellular fractions as previously described (15). For assay of PKC activity, incorporation of ^{32}P from [γ-^{32}P]ATP (NEN) into histone H1 was determined (15). Incubations were conducted in a final volume of 85 μl at 30°C for 30 min. In the final concentrations, the assay mixture contained 25 μM ATP (0.4 μCi), 10 mM Mg acetate, 5 mM β-mercaptoethanol, 50 μg of histone H1, 20 mM HEPES, pH 7.5, phosphatidylserine vesicles (10 μg/ml), and 0.2 mM $CaCl_2$. Incorporation of [^{32}P]phosphate into histone was linear for ≥30 min. The reaction was stopped by addition of 2 ml of ice-cold 5% trichloroacetic acid with 10 mM H_3PO_4. For determination of the radioactivity retained on GF/C glass fiber filters after filtration, the filters were counted in 2 ml of scintillation fluid. PKC activity was determined after subtraction of ^{32}P incorporation in the absence of Ca^{2+} and phospholipids. Data are expressed as picomoles of phosphate incorporated into the substrate per minute per 10^7 cells. Alternatively, the PKC enzyme assay system kit (Amersham Pharmacia Biotech) was used to measure PKC activity purified from subcellular lymphoid fractions according to the manufacturer's instructions. The content of PKC in the samples was calculated by interpolation of the phosphorylation rate (pmol/min) of the samples in a dose-response curve obtained with PKC purified from rat brain. The phosphorylation rate has a linear range up to PKC addition of 10 ng/tube.

Determination of NOS activity. NOS activity was measured by production of [U-^{14}C]citrulline from [U-^{14}C]arginine (Amersham Biosciences) (3). Briefly, cells cultured in the absence or presence of T_3 or T_4 were incubated in 500 μl of Krebs-Ringer bicarbonate solution in the presence of [U-^{14}C]arginine (0.5 μCi). Specificity of NOS activity was evaluated by assays performed in the presence of the NOS blocker N^G-monomethyl-L-arginine monoacetate (L-NMMA), which was previously shown to inhibit enzyme activity in BW5147 cells (3), and the arginase inhibitor L-valine (50 mM). After incubation, the cells were disrupted by sonication (Vibra-cell, Sonics and Materials) in a medium containing 10 mM EGTA, 0.1 mM citrulline, 0.1 mM dithiothreitol, and 20 mM HEPES, pH 7.5. After centrifugation at 20,000 *g* for 10 min, the supernatants were applied to 2-ml columns of Dowex AG 50WX-8 (sodium form, Bio-Rad), and [U-^{14}C]citrulline was eluted with 3 ml of water and quantified by liquid scintillation counter. Recovery of citrulline from Dowex columns was evaluated by addition of [^{14}C]citrulline (2,000 dpm;

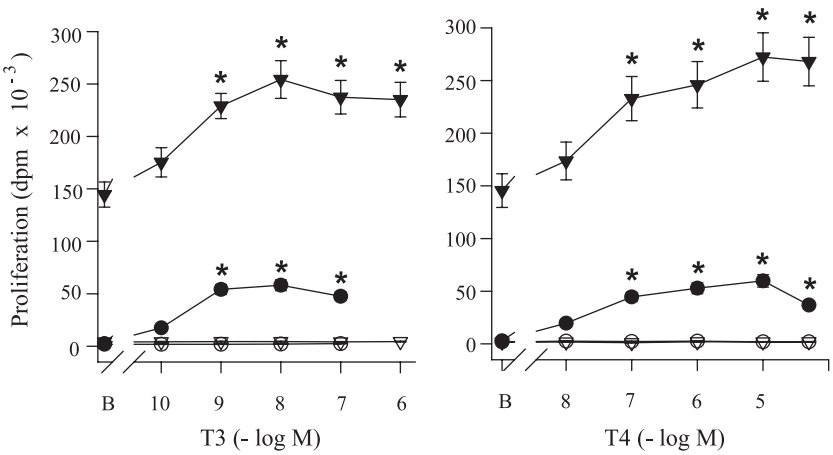

Fig. 1. Effect of thyroid hormones (THs) on normal T lymphocyte proliferation. T lymphocytes were obtained from lymph nodes of normal mice and cultured for 72 h in the absence (open symbols) or presence (filled symbols) of concanavalin A (ConA) mitogen (2 μg/ml) alone (basal, B) or with increasing concentrations of 3,5,3′-l-triiodothyronine (T_3) or thyroxine (T_4). Cells were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics (triangles) or in HyQ medium containing antibiotics (circles). Cell proliferation was determined by [^{3}H]thymidine (TdR) incorporation. Values [disintegrations per minute (dpm)] are means ± SE of ≥5 experiments performed in triplicate. *Significantly different from corresponding basal value ($P < 0.05$).

New England Nuclear) as previously described (38) and was always 85–90%.

Immunoblot analysis of PKC and iNOS isoenzymes. For preparation of whole cell lysate samples from T_3- or T_4-treated or untreated tumor and normal T lymphocytes, cell pellets were dissolved in SDS sample buffer [2% SDS, 10% (vol/vol) glycerol, 62.5 mM Tris·HCl, pH 6.8, 0.2% bromphenol blue, and 10 mM 2-mercaptoethanol] at a final concentration of 10 mg/ml. Equal amounts of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes (15). Nonspecific binding sites in nitrocellulose membranes were blocked with blocking buffer (5% nonfat dry milk containing 0.1% Tween 20 in 100 mM Tris·HCl, pH 7.5, and 0.9% NaCl) for 1 h. The nitrocellulose membrane was subsequently incubated with protein G-purified anti-peptide antibodies to specific PKC isoforms or to iNOS isoenzymes for 18 h. The specific antibodies were as follows: anti-PKC-α, -β_I, -ϵ, and -ζ (Sigma Chemical); anti-PKC-γ and -δ (Pharmingen BRL); and anti-PKC-θ, anti-iNOS Ab, and anti-β_2-microglobulin [as control for protein loading (Santa Cruz Biotechnology)]. Negative controls were incubated overnight in the presence of immunogenic peptides at 2 μg/ml antibody to 1 μg/ml peptide. The membrane was then incubated with a second monoclonal antibody-alkaline phosphatase conjugate (Sigma Chemical) for 1 h. Immunoreactive bands were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. A Full Range Rainbow (Amersham Pharmacia) was used for molecular weight markers. The same samples were run in 15% SDS-polyacrylamide gels and revealed with an anti-β_2-microglobulin antibody (Santa Cruz Biotechnology) as internal control for protein loading. Densitometric analysis was performed by UN-SCAN-IT (version 5.1, Silk Scientific) software.

RT-PCR. Total cellular RNA was extracted from tumor cells using the Aquapure RNA isolation kit (Bio-Rad) following the manufacturer's instructions. RT was performed using the Omniscript kit (Qiagen) with 2 μg of total extranuclear RNA in a 20-μl reaction volume containing 1 μM oligodeoxythymidine-(12–18) (Biodynamics), dNTPs (Promega) at 0.5 mM each, 4 U of Omniscript RT, and 2 μl of 10× RT buffer provided with the kit. The cDNA (2.5 μg) was used for PCR amplification with 1.5 U of *Taq* polymerase (Promega), dNTPs (Promega) at 25 mM each, and 25 mM $MgCl_2$ in a DNA thermal cycler (Progene, Techne) under the following conditions: 95°C for 5 min followed by 32 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were run on a 2% agarose gel (Invitrogen) and stained with ethidium bromide (3). For semiquantification, threefold serial dilutions of template cDNA were done as described elsewhere (30). The primers, which were derived from the published sequences of murine macrophage iNOS cDNA (3, 16) [5′-TCAGACATGGCTTGCCCCTGGA-3′ (forward) and 5′-TGCCCCAGTTTTTGATCCTCACA-3′ (reverse)], generate a 262-bp PCR product. β_2-Microglobulin, used as a housekeeping gene (35), was amplified as indicated using the following primers: 5′-GCTATCCAGAAAACCCCTCAA-3′ (forward) and 5′-CATGTCTCGATCCCAGTAGACGGT-3′ (reverse); it rendered a 300-bp PCR product.

Intracellular delivery of anti-PKC-ζ or anti-PKC-γ antibodies in tumor T cells. Anti-PKC-ζ or -γ antibody against the COOH-terminal region of the corresponding PKC isoenzymes (Sigma Chemical) was introduced into normal or BW5147 tumor T cells after permeabilization with lysolecithin as described elsewhere (15). Anti-PKC-γ, which is absent in BW5147 cells, was used as an irrelevant control antibody. Briefly, BW5147 cells were equilibrated in 7% FBS-containing medium for 24 h and washed with serum-free medium, and then 0.1 ml of glycerol in PBS at 37°C was added. After 6 min on ice, 4 ml of a 1 mg/ml lysolecithin (Sigma Chemical) solution in water was added,

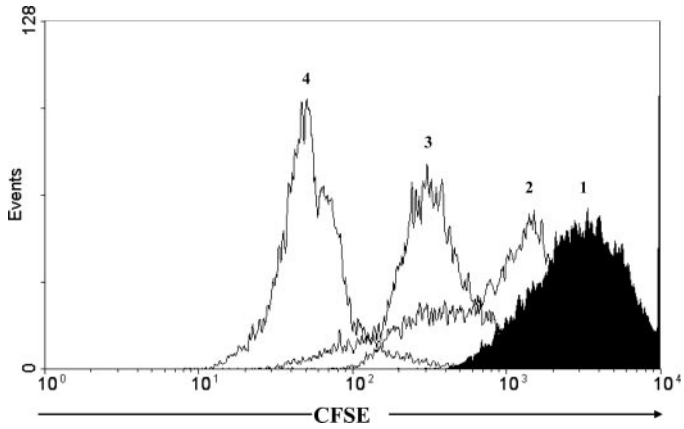

Fig. 3. Carboxyfluorescein diacetate succinimidyl ester (CFSE) analysis of TH action in arrested BW5147 cell growth. FBS-starved BW5147 cells were labeled with CFSE and cultured in FBS-free medium with 10^{-9} M T_3. Cell samples were obtained at 0 h [*peak 1*, mean fluorescence intensity (MFI) = 3,367], 12 h (*peak 2*, MFI = 1,357), 36 h (*peak 3*, MFI = 313), and 72 h (*peak 4*, MFI = 46.5) and measured by flow cytometry. Results are representative of 4 samples from 2 independent experiments.

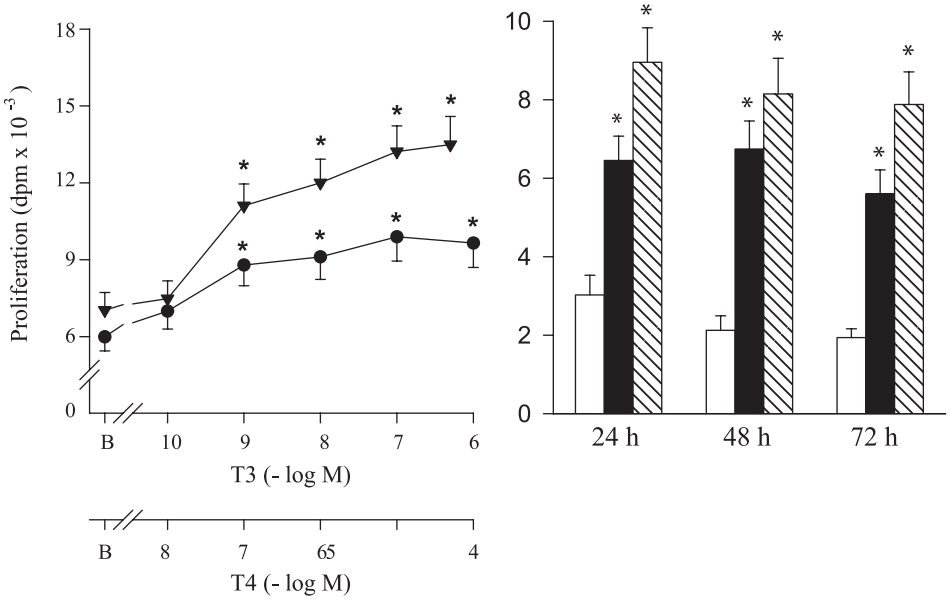

Fig. 2. Concentration-dependent effect of THs on T lymphoma cell proliferation. *Left*: BW5147 cells were cultured in HyQ medium in the absence (basal, B) or presence of increasing concentrations of T_3 (circles) or T_4 (triangles) for 24 h. Proliferation was evaluated by 6-h pulse of [^{3}H]TdR. Values are means ± SE of 4 experiments performed in triplicate. *Significantly different from basal value ($P < 0.05$). *Right*: synchronized BW5147 lymphocytes were cultured in RPMI 1640 without FBS in the absence (open bars) or presence of 10^{-9} M T_3 (filled bars) or 10^{-7} M T_4 (hatched bars) for 24–72 h. Values are means ± SE of 6 independent experiments performed in triplicate. *Significantly different from basal value ($P < 0.01$).

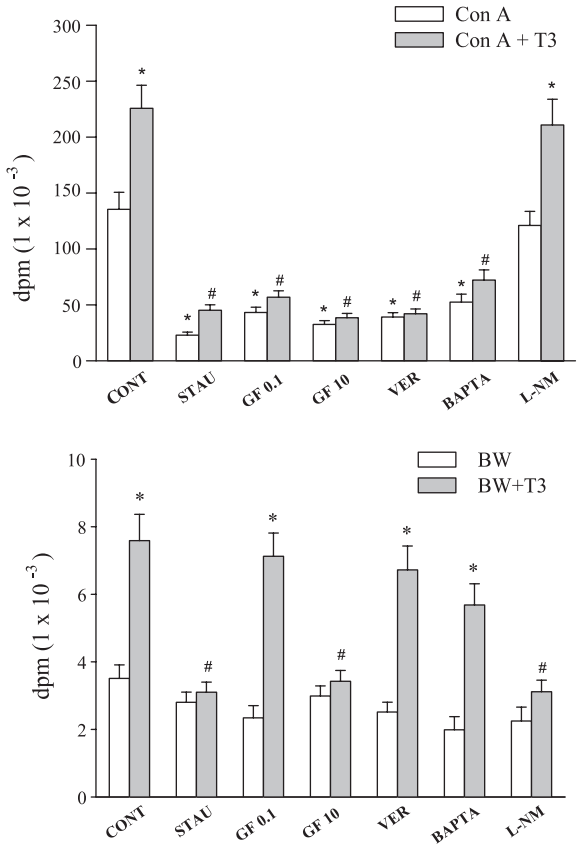

Fig. 4. Effect of enzyme blockers on TH-mediated activation of normal and tumor T lymphocyte proliferation. *Top*: cell proliferation in control T lymphocytes (Cont) cultured with ConA (2 μg/ml) alone (open bars) or ConA + 10^{-9} M T_3 (filled bars) for 72 h, with 0.1 μM staurosporine (STAU), 0.1 or 10 μM GF-109203X (GF), 100 μM verapamil (VER), 25 μM BAPTA, or 500 μM N^G-monomethyl-L-arginine (L-NM) added at the beginning of culture and 90 min before addition of T_3. *Bottom*: cell proliferation in control synchronized BW5147 (BW) cells incubated alone (open bars) or in the presence of 10^{-9} M T_3 (filled bars) for 24 h and treated as described for T lymphocytes (*top*). Values are means ± SE of 3 experiments performed in triplicate. *Significantly different from the Cont value in the absence of hormone ($P < 0.01$). #Significantly different from corresponding control + T_3 value ($P < 0.01$).

and the incubation was continued for 5 min. The cells were brought to 37°C, and 0.1 ml of a dilution of antibody was added. After an additional 10 min at 37°C, during which the cells reseal, 0.1 ml of medium containing 21% FBS was added. Then the cells were returned to the 7% FBS-containing medium, and incubations were continued for 24 h in the presence or absence of T_3 or T_4. Control untreated cells were subjected to the same permeabilization schedule used for intracellular delivery of antibodies in tumor T cells. NOS enzymatic activity was evaluated immediately at the end of incubation (see above).

Drugs. Unless otherwise indicated, all drugs were obtained from Sigma Chemical. Where indicated, enzyme blockers were added at the beginning of cell culture and 90 min before TH addition. The protein kinase inhibitor staurosporine, the selective PKC inhibitor GF-109203X (bisindolylmaleimide), and the intracellular Ca^{2+} blocker BAPTA-AM were dissolved in DMSO. Cell-permeable myristoylated PKC-ζ pseudosubstrate (Myr-PKCζPS) and the extracellular Ca^{2+} blocker verapamil were dissolved in PBS. Stock solutions were freshly prepared before use. Drugs dissolved in DMSO were further diluted (≥1:1,000) in RPMI 1640 to achieve the concentrations indicated in RESULTS.

Statistical analysis. Values are means ± SE. Differences between treatments were analyzed with SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). When multiple comparisons were necessary after ANOVA, the Holm-Sidak test was applied. $P < 0.05$ was considered significant.

RESULTS

Effects of TH on normal and tumor T cell proliferation. To evaluate TH actions on normal and tumor T lymphocyte proliferation, dose-response curves were performed. Hormone effects were studied in resting and mitogen-stimulated normal T lymphocytes. T_3 and T_4 did not induce resting cell proliferation but increased mitogen ConA-induced stimulation at 3 days of culture in a dose-dependent manner (Fig. 1). These effects were observed when cells were maintained in culture conditions usually used for mitogen-induced lymphoid cell growth, i.e., 10% FBS-containing RPMI 1640 (with TH at >1 order below the physiological range), and also in serum-free HyQ medium to avoid possible influence of the hormones present in FBS. Although ConA did not trigger a mitogenic response in HyQ medium, a significant effect of the mitogen was observed in the presence of THs, although to a lesser extent than in RPMI 1640.

The TH-induced increase in BW5147 T lymphoma cell growth in the protein-rich HyQ medium was dependent on the

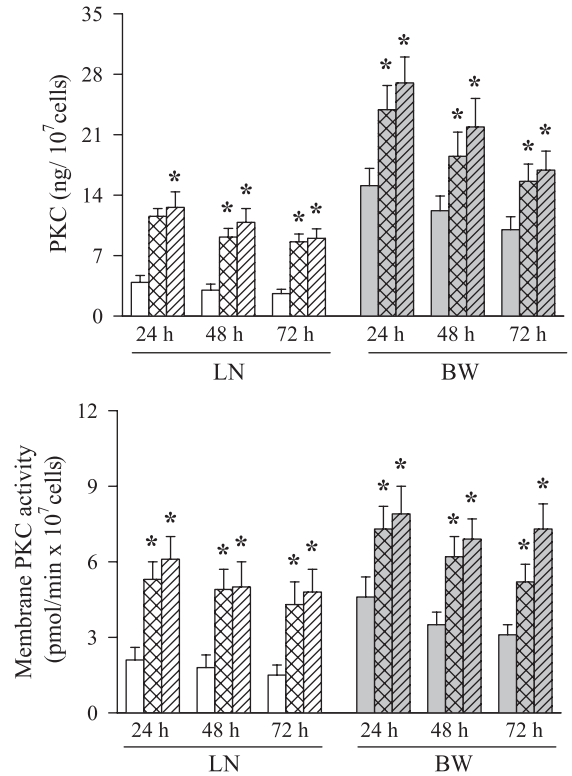

Fig. 5. Effect of TH on PKC activity in normal (LN) and BW5147 (BW) tumor T lymphocytes. Normal T and synchronized BW5147 lymphocytes were cultured for 24–72 h in the absence (control, open bars) or presence of 10^{-9} M T_3 (crossed-hatched bars) or 10^{-7} M T_4 (hatched-dashed bars). *Top*: PKC content. Values are means ± SE of ≥3 experiments performed in duplicate. *Bottom*: PKC activity in purified particulate fractions. Values are means ± SE of 4 experiments performed in duplicate. *Significantly different from corresponding control value ($P < 0.01$).

Table 1. *Effect of T_3 on mitogen-induced translocation of PKC activity in normal T lymphocytes*

	PKC Activity, pmol/min × 10^7 cells		Membrane PKC, % of total activity
	Cytosol	Membrane	
	24 h		
No T_3			
No ConA	14.1±0.9	2.4±0.3	14.5±1.5
ConA	7.1±0.4	9.9±0.7	58.2±4.1*
T_3			
No ConA	46.4±3.2	7.1±0.6	13.2±1.1
ConA	14.4±0.7	40.6±3.1	75.9±6.0*†
	48 h		
No T_3			
No ConA	8.9±0.8	1.5±0.1	14.4±0.7
ConA	4.1±0.3	6.7±0.4	62.0±4.7*
T_3			
No ConA	27.6±2.5	4.4±0.5	13.8±1.0
ConA	6.1±0.5	26.4±1.8	78.8±6.2*†

Values are means ± SE of 3 experiments performed in duplicate. Normal T lymphocytes were cultured in the absence or presence of 3, 5, 3-*t*-triiodothyronine (T_3) 10^{-9} M for 24 or 48 h and stimulated for 15 min with concanavalin A (ConA, 2μg/ml) or left untreated. PKC from cytosolic or particulate fraction was purified, and enzymatic activity was determined by phosphorylation of the specific substrate. Percentages of membrane PKC activity were calculated from total activity (cytosol + membrane). *$P < 0.01$ vs. No ConA. †$P < 0.05$ vs. no T_3.

concentrations of T_3 and T_4 (Fig. 2*A*). The effects of TH were also significant after 24–72 h of culture of arrested BW5147 cells in RPMI 1640 without FBS supplementation (Fig. 2*B*). Proliferative actions became significant in both cell types with 10^{-9} M T_3 or 10^{-7} M T_4, concentrations in the normal range of serum levels in humans and rodents. On the other hand, the effects of TH on the exponentially growing BW5147 cells maintained in 10% FBS supplemented RPMI 1640, in which the cells proliferated maximally, were not evident. To confirm that TH induced BW5147 cell proliferation and to corroborate our [^{3}H]TdR results, arrested cells were stained with CFSE, which binds spontaneously and irreversibly to intracellular proteins. On cell division, CFSE labeling is equally distributed among the daughter cells, which, therefore, contain half as much fluorescent dye as the parental cells (24). BW5147 cells were induced to proliferate by addition of T_3 to quiescent cells, as shown by a progressive decrease in the mean fluorescent peaks with time of culture (Fig. 3). T_3 induced a ~2.5-fold decrease in the CFSE signal over 12 h, corresponding to ~2.7 divisions per day. During the following 36 and 72 h, the signal decreased by another 4.3- and 6.7-fold, corresponding to 2.2 and 1.9 divisions per day, respectively. The slight difference between these results is probably due to the inhibition of growth by cell contact, which is characteristic of hyperproliferative cells. Arrested cells cultured alone, without TH or other growth factors (FBS), displayed similar CFSE signals at 12 h of culture: mean fluorescence intensity (MFI) = 3,429 and 3,220 at 0 and 12 h, respectively. For >12 h, a loss of fluorescence intensity due to a rapid and progressive cell death was observed, e.g., ≥50% dead cells were detected at 72 h of culture.

The effects of TH on lymphocyte proliferation were mainly exerted by T_3, inasmuch as inhibition of T_4 conversion to T_3 by propylthiouracil, at doses that did not affect cell growth, impaired T_4 actions on proliferation of both cell types (data not shown).

For some of the following studies, results with T_4 and T_3 are shown alternately, inasmuch as both hormones induce similar effects.

Effect of different blockers of protein kinases and NOS on TH-mediated stimulation of lymphocyte proliferation. Inasmuch as we previously found a differential participation of PKC and NOS in normal and BW5147 cell proliferation and to ascertain the intracellular signals involved in TH-mediated actions in both cell types, blockers of the above-mentioned enzymes as well as of Ca^{2+} were analyzed. Blockade of PKC by staurosporine or the more selective GF-109203X at a dose that blocks all PKC isoenzymes (10 μM) inhibited normal and tumor T cell proliferation (Fig. 4). However, GF-109203X at a dose blocking only classical PKC isoenzymes (0.1 μM) was effective in abrogating cell growth and T_3-mediated proliferative actions only in normal T lymphocytes. Furthermore, although the extracellular Ca^{2+} blocker verapamil, as well as the intracellular Ca^{2+} blocker BAPTA, efficiently inhibited ConA- and T_3 + ConA-stimulated proliferation, they had little effect on T_3-induced lymphoma growth.

To assess the role of cell-specific pathways for NO in T_3 actions in normal and tumor cells, the effect of the NOS competitive blocker L-NMMA was also determined. Figure 4 also shows that the NOS blocker was able to abrogate T_3 effects on tumor, but not normal, T lymphocytes.

THs increase PKC activity in normal and tumor lymphocytes. To characterize the possible intracellular pathways involved in TH actions on normal and tumor T lymphocyte proliferation, PKC content and membrane-associated PKC activity were measured at different times of cell culture in the presence of T_3 or T_4 at concentrations within the physiological range that affect normal and tumor T cell growth. Incubation with 10^{-9} M T_3 or 10^{-7} M T_4 for 24–72 h increased PKC content of normal and tumor BW5147 T cells (Fig. 5, *top*). A greater activity of PKC was found in particulate fractions of both cell types incubated for similar times with both hormones

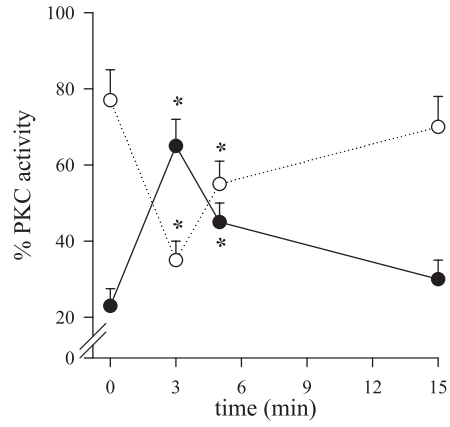

Fig. 6. Short-term effect of T_3 on PKC translocation on BW5147 lymphoma cells. Synchronized BW5147 cells were incubated with 10^{-9} M T_3 for 0–15 min, and PKC activities were measured in purified cytosolic (○) and particulate (●) fractions. Values are means ± SE of 3 experiments performed in duplicate. *Significantly different from control values before the addition of T3, *time 0*; $P < 0.05$.

than in untreated controls (Fig. 5, *bottom*), with no modification in cytosol-to-membrane ratio. Short-term incubation with T_3 induced a rapid and transient translocation of PKC to cell membranes on tumor lymphocytes (Fig. 5), but 15 min of incubation had no effect on normal T cells (data not shown). T_4 exerted similar effects.

Results in normal T cells indicate that the TH-mediated increment in PKC is not sufficient to stimulate cell proliferation in the absence of the additional stimulus of the mitogen, inasmuch as THs were not able to stimulate the proliferation of resting normal T lymphocytes. ConA induced a higher translocation of PKC activity to particulate fractions in cells previously incubated for 24–48 h with T_3 than in untreated T lymphocytes (Table 1). However, in tumor T lymphocytes, greater PKC content and membrane-associated activity, as well as rapid PKC translocation to the cell membrane in the presence of THs (Figs. 5 and 6) would be related to cell growth, inasmuch as no other addition was necessary for proliferation of synchronized cells.

THs induce a differential modulation of PKC isoenzymes in normal and tumor T lymphocytes. To further characterize PKC differential participation in TH actions in normal and tumor T lymphocytes, the PKC isoenzyme pattern was analyzed in both cell types before and after T_3 or T_4 treatment. Both hormones induced a differential increase in individual PKC isoenzymes in ConA-stimulated and BW5147 T lymphocytes (Fig. 7). The conventional PKC-α and PKC-β isoforms were increased in normal cells, whereas the atypical PKC-ζ isoenzyme was increased in T lymphoma cells. PKC-γ (absent in both cell types), PKC-ϵ, and PKC-θ were unchanged (data not shown) were unchanged. Thus THs were not able to change the PKC pattern normally expressed in both cell types.

Stimulation of NOS activity in tumor T lymphocytes by TH: involvement of iNOS. NOS activity is crucial for BW5147 cell proliferation (3) and occurs downstream from PKC in tumor, but not normal, T lymphocytes (15), so the effect of THs on NOS activation was studied in both cell types. T_3- and T_4-induced NOS activation in normal lymphocytes was different from that in BW5147 lymphocytes. Proliferative actions of THs were accompanied by an increment in NOS activity in lymphoma, but not in normal T cells (Fig. 8). NOS activity was determined by the formation of radiolabeled citrulline from [^{14}C]arginine in normal or tumor T cells cultured in the absence or presence of THs and in the presence of L-valine, a blocker of arginases. Results from experiments performed in the absence of L-valine were similar (data not shown) to those obtained in the presence of the arginase blocker, so involvement of the arginase pathway in TH-mediated actions in tumor cells could be ruled out.

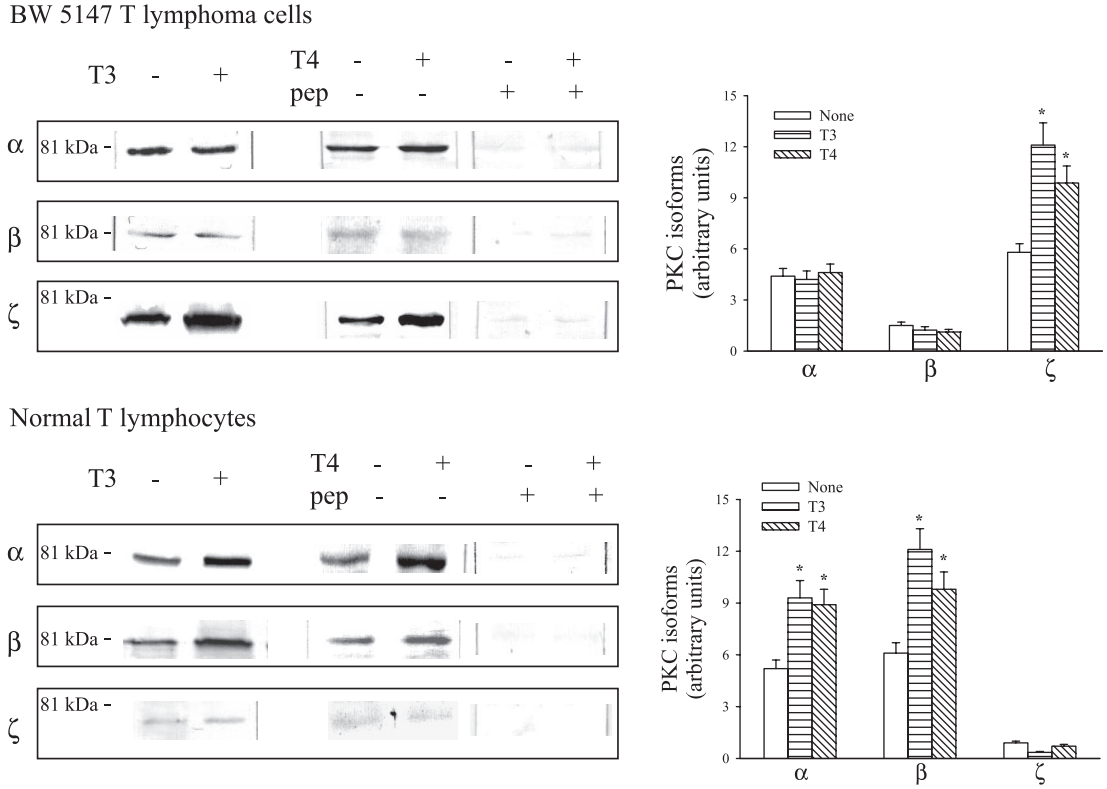

Fig. 7. Effect of THs on individual PKC isoenzymes in normal and tumor T cells. PKC isoforms expressed in BW5147 cells and ConA-stimulated T lymphocytes cultured in RPMI 1640 in the absence (−, control) or presence (+) of 10^{-9} M T_3 or 10^{-7} M T_4 for 24 or 72 h were analyzed by Western blot. Specificity of anti-PKC antibody was verified by preincubation of each antibody in the presence of the corresponding blocking peptide (pep) against which the antibody was raised (data correspond to T_4-treated or untreated cells). Results are representative of 4 independent experiments. Densitometric results of Western blot analysis in the absence (open bars) or presence of T_3 or T_4 (horizontal-lined and hatched bars, respectively) represent relative level for each PKC isoenzyme. Values are means ± SE. β_2-Microglobulin bands obtained to check protein loading were similar for all cases (data not shown). *Significantly different from corresponding control ($P < 0.05$).

Inasmuch as we previously demonstrated that iNOS is directly related to BW5147 lymphoma cell growth (3), we investigated whether this isoenzyme was modified by TH treatment. Preincubation of cells with T_4 leads to an increased protein expression of iNOS (Fig. 9, *A* and *B*). Semiquantitative RT-PCR analysis showed significantly higher iNOS transcription in tumor cells (Fig. 9).

PKC-ζ-dependent modulation of basal and T_4-induced NOS activity. We previously showed that atypical PKC-ζ expression is associated with NOS regulation in BW5147 T lymphoma cells (15). Therefore, the relation between this PKC isoform and TH-induced NOS activity was analyzed. Table 2 shows that blockade of PKC by staurosporine or by 10 μM, but not 0.1 μM, GF-109203X, inhibited basal and T_4-mediated increases in NOS activity. Moreover, intracellular delivery of an anti-PKC-ζ, but not the irrelevant anti-PKC-γ, antibody and preincubation with the specific cell-permeable Myr-PKCζPS abrogated basal, as well as T_4-mediated, stimulation of NOS activity (Table 2). Myr-PKCζPS also decreased the basal and T_4-mediated increase in iNOS mRNA expression in tumor T cells (Fig. 9*D*). Myr-PKCζPS also inhibited basal (77 ± 7% inhibition), as well as T_3- and T_4-induced (82 ± 9 and 90 ± 8%, respectively), proliferation in BW5147 cells.

Table 2. *Effect of PKC-ζ blockade on T_4-mediated activation of NOS in BW5147 cells*

	NOS Activity; pmol/10^7 cells	
	Basal	T_4 stimulated
Untreated	95±8	147±15*
Staurosporine (0.1 μM)	33±3†	42±3†
GF-109203X		
0.1 μM	86±8	132±12*
10 μM	31±4†	46±5†
Anti-PKC-ζ	40±4†	49±5†
Anti-PKC-γ	114±10	165±15*
Myr-PKCζPS (50 μM)	47±5†	54±6†

Values are means ± SE of 3 experiments. Myr-PKCζPS, myristoylated PKC-ζ pseudosubstrate. BW5147 cells were cultured alone (basal) or in the presence of 10^{-7} M thyroxine (T_4) for 24 h, with blockers added 90 min before addition of T_4. Cells were also permeabilized and treated with a 1:100 dilution of anti-PKC-ζ or anti-PKC-γ antibody. Nitric oxide synthase (NOS) activity was determined at the end of the culture in the presence of the arginase blocker L-valine (50 mM). Permeabilization has no effect on NOS activity, in as much as there was no statistically significant difference between cells treated with T_4 in the absence of antibody and unpermeabilized cells (97 ± 10 pmol/10^7 cells). *$P < 0.001$ vs. basal. †$P < 0.01$ vs. untreated.

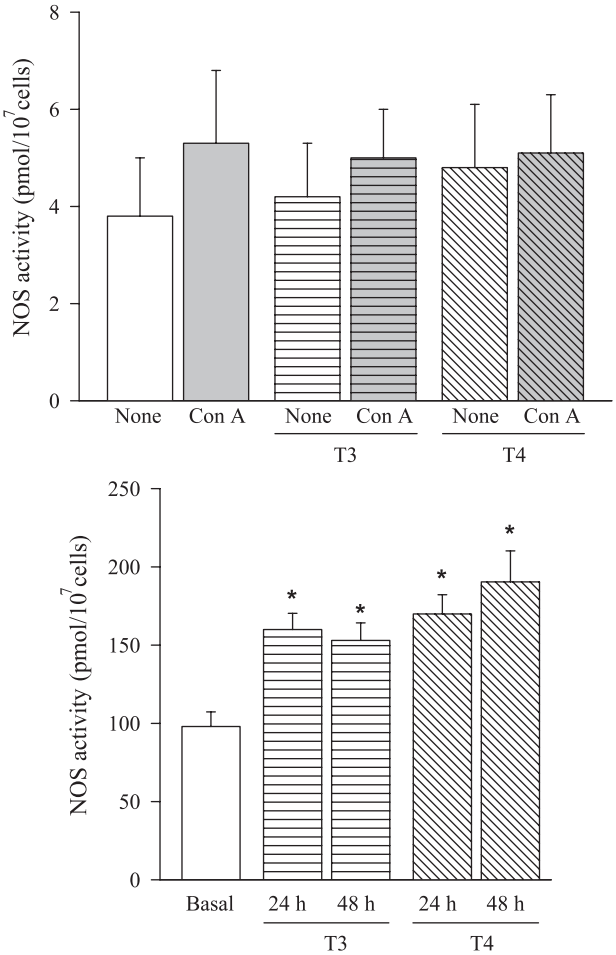

Fig. 8. TH-induced increase of nitric oxide synthase (NOS) activity in BW5147 cells, but not in normal T lymphocytes. *Top*: NOS activity from conversion of [^{14}C]arginine to [^{14}C]citrulline in resting normal T lymphocytes (none) or after stimulation with ConA (2 μg/ml) in the absence or presence of 10^{-9} M T_3 or 10^{-7} M T_4 for 72 h. *Bottom*: NOS activity in BW5147 cells cultured in the absence (basal) or presence of 10^{-9} M T_3 or 10^{-7} M T_4 for 24 or 48 h. Values are means ± SE of 4 experiments performed in duplicate. *Significantly different from corresponding basal value ($P < 0.01$).

DISCUSSION

We have analyzed, for the first time, the effect of THs on tumor T lymphocyte, compared with normal T cell, proliferation and the involvement of the PKC isoenzyme and NOS in both cell types. We found that T_3 and T_4 stimulated tumor, but not resting normal, T lymphocyte proliferation, although these THs could increase normal T cell responses to mitogen, as previously described by Keast and Taylor (19). These effects were TH concentration dependent, as indicated by the dose-response curves of [^{3}H]TdR incorporation. TH effects were not evident in BW5147 cells proliferating maximally in optimal culture conditions (RPMI 1640 containing 10% FBS), probably because of the presence of the other growth factors in FBS that overlap TH actions. However, induction of cell proliferation in BW5147 cells was also confirmed by CFSE-labeling experiments, which show that THs are able to induce cell division in arrested cells.

PKC and NOS activities play important roles in the regulation of cell functions and were demonstrated to play differential roles in the control of normal and tumor T cell proliferation (15). Therefore, their participation in TH actions was also analyzed. PKC inhibitors, i.e., staurosporine and GF-109203X, a potent inhibitor of classic PKC but a weak inhibitor of Ca^{2+}-independent isoforms, were able to block TH effects on cell proliferation in both cell types. However, in tumor cells, high doses of GF-109203X were needed to abrogate TH-induced proliferation. These results indicate that PKC isoforms would have different hierarchical participation in TH-mediated effects on normal and tumor T cell activation.

Additionally, 24–72 h of incubation with T_3 or T_4 induced an increment in the total content of PKC, as well as an increase in membrane-associated PKC activity, in normal and tumor T

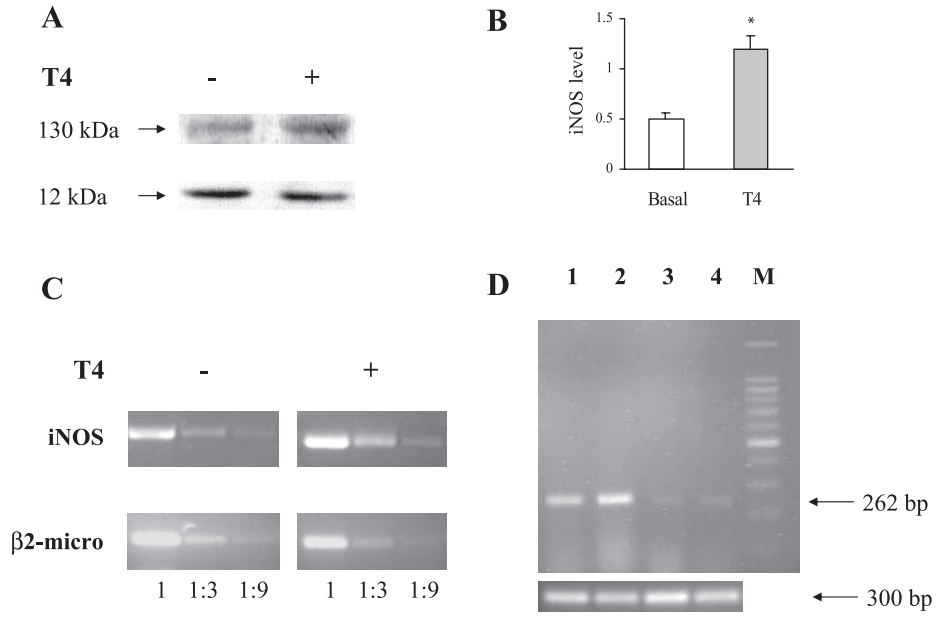

Fig. 9. Regulation of inducible NOS (iNOS) expression by T_4 and effect of PKC-ζ blockade. *A*: Western blot of iNOS expressed in BW5147 cells cultured for 24 h in the absence (−, control) or presence (+) of T_4. Specificity of anti-iNOS antibody, revealing a 130-kDa band, was verified by preincubation of the antibody with the corresponding blocking peptide (data not shown). Relative amounts of β_2-microglobulin (12-kDa band) for the same samples are also shown. Results are representative of 4 independent experiments. *B*: iNOS levels corresponding to densitometric analysis of bands obtained in the absence (open bar) or presence (filled bar) of T_4. Each value was calculated relative to corresponding β_2-microglobulin value. $^*P < 0.05$ vs. basal. *C*: mRNA levels of iNOS and β_2-microglobulin (β_2-micro) housekeeping gene in total RNAs prepared from cells cultured as described in *A* and determined by semiquantitative RT-PCR with 3-fold serial dilution of template cDNA. Blots are representative of 3 experiments with similar results. *D*: effect of PKC-ζ blockade on iNOS mRNA expression. mRNA from tumor T lymphocytes was cultured in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of T_4 and 50 μM myristoylated PKC-ζ pseudosubstrate (*lanes 3* and *4*). M, DNA molecular weight marker. Results are representative of 3 experiments. Labeled bands (262 and 300 bp) correspond to iNOS and β_2-microglobulin, respectively.

lymphocytes. In normal cells, the TH-mediated increase in PKC is not sufficient to stimulate the proliferation of resting normal T lymphocytes, inasmuch as it is known to require the additional signal of an increase in cytosolic Ca^{2+} (8). The extracellular Ca^{2+} blocker verapamil, as well as the high-affinity intracellular Ca^{2+} chelator BAPTA, inhibits ConA and TH actions on ConA-induced proliferation of normal cells but had no significant effect on the proliferation of BW5147 cells. In these cells, PKC activity in the presence of TH would be related to cell growth, inasmuch as no other addition is necessary for proliferation of arrested cells. On the basis of these results and the higher ConA-mediated rapid translocation of PKC to particulate fractions in T_4-preincubated T lymphocytes than untreated controls, the possibility that PKC is upregulated by a genomic action of TH emerges. Moreover, the rapid and transient TH-mediated translocation of PKC activity only in tumor cells suggests that THs are able to exert nongenomic actions in tumor cells as well.

THs are well-known modulators of signal transduction, and some experimental evidence demonstrates that TH mediated genomic and nongenomic actions on PKC signaling in different cell types. Krasilnikova et al. (21) showed that drug-induced hypothyroidism decreases T_3 and T_4 serum levels and total PKC activity in the liver. In contrast, under hypothyroid conditions, PKC expression and activity were upregulated in rat ventricular myocytes (34, 36). Alisi et al. (1) demonstrated that T_3 and T_4 regulated cell growth in chick embryo hepatocytes through the activation of PKC-α and MAPK. Moreover, PKC is critical to the mechanism by which THs rapidly induce phosphorylation and nuclear translocation of mitogen-activated protein kinase (22) and, subsequently, potentiate the antiviral and immunomodulatory actions of IFN-γ in cultured HeLa cells (23).

To further characterize TH-mediated PKC differential modulation of normal and tumor T lymphocyte proliferation, the PKC isoenzyme pattern was analyzed in both cell types before and after TH treatment. We found that T_3 and T_4 were not able to modify the PKC profile of each cell type. However, in ConA-treated normal T lymphocytes, both hormones increased the levels of Ca^{2+}-dependent PKC-α and PKC-β, whereas the Ca^{2+}-independent atypical PKC-ζ was increased in tumor T cells. These are the main isoenzymes involved in the modulation of proliferation of each cell type (15). Changes in levels of PKC isoenzyme expression by THs were demonstrated in other cell types. Rybin and Sternberg (34) showed a T_3-dependent reduction of PKC-α and PKC-ε in neonatal myocytes and only PKC-ε in adult myocytes, but not PKC-δ or PKC-ζ. Differences in PKC-α levels were induced by hypothyroidism and T_4-replacement therapy in rat liver (26), and T_3 was described to increase PKC-α, but not PKC-β_2 or PKC-ε, in human umbilical vein endothelial cells (4). Taken together, these findings and our experimental results suggest a role for regulation of PKC isoform expression in TH actions in different cell types and tissues.

Differential actions of TH were also found between normal and tumor BW5147 lymphocytes, inasmuch as proliferative actions of TH were accompanied by an increase in NOS activity in lymphoma, but not normal, T cells. Furthermore,

TH-induced NOS activation in BW5147 cells was accompanied by an increase in protein and mRNA levels of iNOS. TH modulation of NOS activity in different tissues has been demonstrated experimentally. The hyperthyroid state was found to enhance NO release by phagocytic cells from bronchoalveolar lavage in adult rats compared with euthyroid controls (18). Three days of treatment with T_3 had similar effects on liver NOS activity; these effects were reversed to control values after 3 days of hormone withdrawal (13). Napoli et al. (29) showed a marked basal vasodilatation that was largely accounted for by excessive endothelial NO production in hyperthyroid patients that was corrected by return to the euthyroid state.

To investigate the relation between NOS and PKC activities, the effects of PKC blockers were analyzed. Staurosporine and GF-109203X at concentrations that block all PKC isoforms, but not at concentrations that block only conventional and novel PKC isoenzymes (40), were able to abrogate basal and TH-induced activation of NOS. Furthermore, intracellular delivery of an anti-PKC-ζ antibody and preincubation with the specific Myr-PKCζPS at a concentration that was demonstrated to inhibit PKC-ζ activity (12, 32) inhibited basal and TH-mediated stimulation of NOS. These results strongly suggest a role for PKC-ζ as a possible downstream mediator of TH-induced NOS activation. This was confirmed by the decrease in iNOS mRNA levels in basal and T_4-stimulated BW5147 cells induced by preincubation with Myr-PKCζPS. Furthermore, Myr-PKCζPS also inhibited basal and TH-mediated proliferation of BW5147 cells according to our previous observations of the effect of intracellular delivery of an anti-PKC-ζ antibody on basal growth (15). In support of our results, overexpression of PKC-ζ was demonstrated to markedly increase iNOS expression in rat mesangial cells (28) and to be involved in inducing iNOS expression in stimulated murine RAW264.7 cells (42). Moreover, another Ca^{2+}-independent PKC isoenzyme, PKC-ϵ, was shown to upregulate iNOS gene and protein expression in the heart at late preconditioning phases (37) and to be critical for iNOS induction in murine macrophages (6).

Finally, we have shown, for the first time, a proliferative action of THs involving different PKC and NOS activities in normal and tumor T lymphocytes. In normal cells, the effects are mediated via activation of conventional PKC isoforms and required the additional signal of a mitogenic stimulus, without affecting NOS activity. These effects could be interpreted as a controlled response and would explain the studies that support the increased immune responses in hyperthyroid conditions (20).

In BW5147 cells, atypical PKC-ζ is the main isoenzyme involved in TH actions, together with the participation of increased NOS activity. Our results show a differential role of NOS activity in TH-mediated actions in normal and tumor cells.

TH administration has been indicated in selected cases of congenital hypothyroidism that result in severe immunodeficiency (33). TH deficiency may significantly alter the balance between malignant tumor viability/growth and cell death (17). A better understanding of the biochemical mechanisms induced by THs in the control of lymphocyte proliferation would help characterize the physiopathological role of THs in normal and hyperproliferative processes and provide a basis for regulation of THs in processes that accompany immunological abnormalities.

ACKNOWLEDGMENTS

The authors thank Maria Rosa González Murano for technical support and Patricia Fernández for secretarial assistance.

GRANTS

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Grant PIP-CONICET 02720 and University of Buenos Aires Grant UBACYT B041.

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