Tri-iodothyronine induces proliferation in cultured bovine thyroid cells: evidence for the involvement of epidermal growth factor-associated tyrosine kinase activity

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Abstract

The effects of the tri-iodothyronine (T3) secreted by thyroid cells on the growth of the thyrocyte are poorly known. In this study we analyzed the effects of T3 on the proliferation of bovine thyroid follicles in primary culture previously depleted of endogenous T3. Cellular deoxiribonucleic acid (DNA) synthesis, determined by [3H]thymidine incorporation, was stimulated by T3 (0·1–5·0 nM) for 24 h in a concentration-dependent fashion with a maximal effect at 1·0 nM T3 (P<0·01). This T3 action was time-dependent when assayed from 12 to 72 h. The induction of mitogenic activity was corroborated by the increase in proliferating cell nuclear antigen (PCNA) measured by Western blot analysis. PCNA increased after treatment with T3 (0·1–5·0 nM) in a concentration-dependent manner. Since T3 modifies the activity of growth factors whose actions are mainly mediated by tyrosine kinase (TK) activation in diverse cellular types, we assayed the effects of genistein, a general TK inhibitor, and tyrphostin A25, a specific epidermal growth factor (EGF)-receptor (EGFR)-dependent TK activity inhibitor, on the proliferative effects of T3. The T3-induced [3H]thymidine incorporation was inhibited by both agents in a concentration-dependent manner. A significant increase in the total TK activity measured in cellular protein extracts was induced by 0·5 and 1·0 nM T3 (P<0·001). Tyrosine phosphorylation of the EGFR was also stimulated by T3 (P<0·001) with no change in the EGFR expression as determined by Western blot analysis. Both, the T3-stimulated [3H]thymidine incorporation and the TK activity were inhibited by an anti-mouse EGF antibody. These results lead us to propose that T3 could operate as a proliferative agent in bovine thyroid cells through a mechanism involving an autocrine/paracrine EGF/EGFR-dependent regulation.

Introduction

Several authors investigated the T3 effects on thyroid cells with controversial results. It has been reported that thyroid tissue takes up and dehalogenates exogenous thyroid hormone (TH) rapidly (Pisarev 1985). Other observations indicate that TH can inhibit thyroid cell function both in vivo and in vitro (Juvenal et al. 1981, Akiyugu et al. 1992, Spitzweg et al. 1999). The presence of nuclear T3 receptors (T3R) in thyroid cells from some species has been well established (Erkenbrack & Rosenberg 1986, Pisarev et al. 1986, Nakamura & Imura 1988, Brännegård et al. 1994), raising the possibility that T3R could participate in the regulation of gene expression by T3 in thyroid cells.

Previous results indicate that T3 was able to induce cell proliferation in the rat thyroid cell line FRTL-5 and in benign and malignant thyroid tumors (Akiyugu et al. 1992, Nagy et al. 1999). However, the mechanisms involved in the proliferation of these cells probably differ from those in normal thyroid cells, a possibility that emphasizes the interest in the study of the proliferative actions of T3 on thyroid cells in primary culture. Several growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I) and others, whose actions are mediated by tyrosine kinase (TK) receptors, have been shown to be important for thyroid cell proliferation in all the model systems tested so far (Dumont et al. 1992, Bidey et al. 1999). Some of these factors are reported to be involved in thyroid folliculogenesis and goitrogenesis (Nilsson 1995, Roger et al. 1997, Bidey et al. 1999). Moreover, a thyroid hormone-dependent increase in the levels of immunoreactive EGF and EGF-messenger ribonucleic acid (mRNA) in the mouse thyroid gland has been reported, suggesting that T3R could modulate the thyroid function through EGF (Osawa et al. 1991, Sheflin et al. 1993).
The objective of this study was to examine the actions of T₃ on the proliferation of bovine thyroid cells previously depleted of endogenous T₃ and evaluate the participation of EGF receptor (EGFR) activation in the T₃ effect. T₃-induced DNA synthesis was demonstrated when cell proliferation was assessed by [³H]thymidine incorporation and corroborated by the expression of the proliferating cell nuclear antigen (PCNA) as a marker of mitotic activity (Iatropoulous & Williams 1996). Genistein and tyrphostin A25, a general inhibitor of protein tyrosine phosphorylation and an EGFR-specific TK inhibitor, respectively, were used to determine whether tyrosine phosphorylation was involved in the T₃-induced DNA synthesis. We also aimed to analyze the effect of T₃ on the induction of TK activity, EGFR expression and EGFR tyrosine phosphorylation as well as the action of an anti-EGF antibody on the T₃-proliferative effect. Our results demonstrated that T₃ acts as a proliferative agent in bovine thyroid cells and support the contention that EGFR-mediated tyrosine phosphorylation may play an important role in T₃-induced proliferation.

Materials and Methods

Materials

Tritiated thymidine ([methyl-³H]thymidine), [γ³²P]ATP and the Renaissance NEL-1 immuno-chemoluminescence kit, were from DuPont NEN (Boston, MA, USA). Human epidermal growth factor (hEGF) was from Calbiochem (La Jolla, CA, USA). Primary sheep polyclonal anti-human EGFR antibody, Dulbecco:Ham F12 (1:1) serum-free tissue culture medium modified by Coon, and Protein TK Assay System were from Gibco BRL (Gaithersburg, MD, USA). Rabbit polyclonal anti-human PCNA (FL-261), and monoclonal mouse anti-phosphotyrosine (PY20) primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine polyclonal anti-mouse EGF antibody, not cross-reacting with transforming growth factor α (TGF-α), and tissue culture grade reagents were purchased from Sigma Chemical Co except where indicated.

Primary culture of bovine thyroid follicles and T₃ depletion

Primary cultures of bovine thyroid cells were obtained according to the protocols described previously (Roger et al. 1986, Costamagna et al. 1998) with modifications. Briefly, bovine thyroid tissues obtained from a local abattoir were digested with collagenase type IV (0.3 mg/ml), DNase I (5 µg/ml) and CaCl₂ (1.0 mM) for 2 h at 38 °C and cultured in suspension. The serum-free culture medium was supplemented with bovine transferrin (5 µg/ml), insulin (10 µg/ml), glutamine (2 mM), and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin, 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B). Cells were cultured in a controlled atmosphere of 95% air–5% CO₂ at 37 °C. Endogenous T₃ was depleted by changing the culture medium every 24 h for 7 days. T₃ concentration in the culture medium was determined daily by radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA, USA). Agents to be tested were incorporated on day 7 of culture in triplicate dishes for the time indicated. The cell viability, as assessed by the Trypan Blue exclusion test, ranged from 80–90% (Freshney 1987).

¹H]thymidine incorporation

The incorporation of [³H]thymidine (20 Ci/mmol) into cellular DNA was measured according to protocols described (Kraiem et al. 1990, Sheffield 1998) with minor modifications. Briefly, T₃-depleted thyroid follicles were treated with the different agents for the time indicated plus 2-5 µCi [³H]thymidine during the last 24 h. When the effect of T₃ was studied for 12 or 24 h, [³H]thymidine and T₃ were added together. Excess radioactivity was eliminated by washing the cells with phosphate-buffered saline (PBS: 9·1 mM NaH₂PO₄, 1·7 mM Na₂HPO₄, 150 mM NaCl, pH 7·4) and the DNA precipitated by incubation at 4 °C for 30 min with 1 vol 20% trichloroacetic acid (TCA). The pellet was washed with 10% TCA, dried at 50 °C and dissolved in NCS. Precipitable DNA radiation was measured by scintillation counting and related to DNA content. Total cellular DNA was quantified according to Burton (1956).

Total cellular protein extract preparation

The expression of PCNA, EGFR and EGFR tyrosine phosphorylation was determined in protein extracts from T₃-depleted thyroid cells treated with T₃ (0·1–5·0 nM) by Western blot analysis prior to immunoadsorption of PCNA and EGFR with specific antibodies, according to standard procedures (Sambrook et al. 1989), with modifications. Briefly, thyroid cells were rinsed twice with cold PBS and resuspended in cold lysis buffer (LB: 10 mM HEPES pH 7·4, 1 mM MnCl₂, 10 mM MgCl₂, 0·1 mM EGTA, 0·5% Triton X-100) in the presence of protein phosphatase and protease inhibitors (40 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride, 5 µg/ml leupeptine, 5 µg/ml aprotinin). Total cellular protein extracts were obtained by successive passages through a syringe and needle, and centrifuged at
13 000 g. The protein content of the supernatant was determined according to Lowry et al. (1951).

PCNA and EGFR immunoadsorption

PCNA and EGFR were immunoadsorbed by incubating aliquots (200 µg protein) of the extracts for 2 h at 4 °C with rabbit polyclonal anti-human recombinant PCNA antibody (1:100) or polyclonal sheep anti-human EGFR antibody (1:750), respectively. The immunocomplexes were recovered from the mixture by incubation with protein G coupled to sepharose 4B overnight at 4 °C with gentle agitation and centrifuged at 10 000 g for 10 min.

Determination of expression of PCNA, EGFR and EGFR tyrosine phosphorylation by Western blot analysis

Immunoadsorbed proteins were carefully washed 3 times with cold LB, resuspended in 20 µl Tris–HCl (1 M) pH 6.8 and electrophoresed (Mini-Protean II, Electrophoresis Cell, Bio–Rad) in 7.5–10% polyacrylamide gels with 1% SDS (SDS-PAGE) in parallel with prestained molecular weight markers. Proteins were transferred to a nitrocellulose membrane for 70 min at 100 V in a Trans-Blot cell (Mini Trans-Blot, Electrophoretic Transfer Cell, Bio-Rad). To detect PCNA and EGFR, antibodies at 1:600 and 1:2000 dilution, respectively, were used. Tyrosine-phosphorylated EGFR was detected by a primary mouse monoclonal anti-phosphotyrosine antibody (PY20) at 1:500 dilution. Goat anti-rabbit IgG (1:2000), rabbit anti-sheep IgG (1:10 000) and sheep anti-mouse IgG (1:400) were used as HRP-conjugated secondary reagents. The HRP reaction was developed by immunchemoluminiscence. The spots were analyzed and semi-quantified densitometrically at 500 nm (Shimadzu Dual-Wavelength Chromato Scanner CS-930).

Tyrosine kinase activity

Aliquots from total cellular protein extracts (5 µg protein) were incubated in 20 µl kinase buffer (30 mM HEPES pH 7.4, 10 mM MgCl₂, 0.02 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.025 mg/ml BSA, 0.15% v/v Nonidet-P 40, 0.07 mM Na₃VO₄, 0.06 mM ATP) in the presence of 0.5 mM specific TK substrate peptide RR–SR, and 1.0 µCi [γ³²P]ATP (6000 Ci/mmol) at 30 °C for 30 min and the reaction was stopped with cold 10% TCA. Aliquots from the supernatant were spotted onto phosphocellulose filters and rinsed twice with 1% acetic acid and twice with water before quantification in a liquid scintillation counter. The results were expressed as pmol P incorporated in 30 min of reaction. Preliminary studies indicated that the reaction was linear from 1 to 15 µg of thyroid protein extracts. Controls for TK activity were run as described previously (Sheffield 1998). TK activity conducted in the absence of the substrate peptide was <15%, indicating an adequate specificity of the reaction.

Statistical analysis

The analysis of multiple intergroup differences in each experiment was conducted by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls’ test. P<0.05 was considered statistically significant. Except when indicated, all values were obtained from three independent experiments in which at least triplicate samples were assayed.

Results

T₃ depletion from bovine thyroid follicles in primary culture

In order to obtain follicular cells depleted of endogenous T₃, the culture medium was changed every 24 h for 7 days. Table 1 shows that daily changes of culture medium effectively reduced the total T₃ concentration after 7 days of culture, indicating that the culture system had reached optimal conditions to evaluate the exogenous T₃ action.

<table>
<thead>
<tr>
<th>Culture day</th>
<th>T₃ (nM/µg DNA)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.84 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>0.78 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>0.66 ± 0.14</td>
</tr>
<tr>
<td>4</td>
<td>0.33 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

The culture medium of thyroid follicles was changed daily. The T₃ concentration (nM) was determined by radioimmunassay in aliquots of culture medium and related to the total cellular DNA (µg) content. Each value represents the mean ± s.d. (n=3) of a pool of triplicate samples from three independent experiments.

Stimulation of [³H]thymidine incorporation and PCNA expression by T₃

Bovine thyroid cell proliferation was determined by measuring the incorporation of [³H]thymidine into cellular DNA and by the PCNA expression by Western blot analysis.

The effect of T₃ (0·1–5·0 nM) on [³H]thymidine incorporation in T₃-depleted bovine thyroid cells is shown in Fig. 1. A concentration-dependent increase with a maximal stimulation at 1·0 nM T₃ was observed. No significant effect was registered at 5·0 nM T₃. Figure 2

Table 1 Depletion of endogenous T₃ in culture medium of bovine follicles
Inhibition of T3-induced \[^{3}H\]thymidine incorporation by cycloheximide

To investigate the involvement of protein synthesis in the effect of T3 on \[^{3}H\]thymidine incorporation, the action of cycloheximide was analyzed. The addition of 1-0 µg/ml cycloheximide significantly inhibited the 1-0 nM T3-stimulated \[^{3}H\]thymidine incorporation, whereas the control value (Fig. 4) or the cell viability (data not shown) were not altered.

Inhibition of T3-induced \[^{3}H\]thymidine incorporation by TK inhibitors

The action of genistein, a general TK inhibitor, and tyrphostin A25, claimed to be an EGFR-specific inhibitor (Levitzky & Gazit 1995, Partik et al. 1999), were assayed on the T3-induced \[^{3}H\]thymidine incorporation. Genistein (1-10 µM) reduced the T3-stimulated \[^{3}H\]thymidine incorporation in a concentration-dependent fashion (Fig. 5). A similar concentration-dependent inhibition was exerted by tyrphostin A25 (1-10 µM) (Fig. 6). Bovine thyroid cells incubated with the highest concentration of genistein or tyrphostin A25 (10 µM) used in the experiments had no effect on cell viability as assessed using the Trypan Blue exclusion test.

Stimulation of total TK activity by T3

Results from experiments with TK activity inhibitors suggested that TK activity could play some part in the action of T3 on DNA synthesis. To check this hypothesis we measured the total TK activity in cellular protein extracts from T3-depleted bovine thyroid cells treated with different concentrations of T3. As shown in Table 2, a significant increase of the TK activity was observed. The effect was concentration dependent with a maximum at 1-0 nM T3. Interestingly, a measurable basal activity was registered in the protein extract.

From the data obtained with tyrphostin A25, a mediation of the EGFR in the TK activation induced by T3 was suspected. To estimate the involvement of EGFR in the T3-increased TK activity, the effect of anti-EGFR antibody was assayed. The T3-induced TK activity was progressively reduced by increasing concentrations of the anti-EGFR antibody, suggesting that the TK activity observed in response to T3 was predominantly due to the EGFR activation (Table 3).

Effect of T3 on the expression of EGFR and EGFR–tyrosine phosphorylation

To further investigate the involvement of EGFR in the induction of TK activity and DNA synthesis observed in response to T3, changes in EGFR expression were analyzed by Western blotting of the immunoadsorbed EGFR.

Absence of an iodide effect on \[^{3}H\]thymidine incorporation

The role of iodide in thyroid cell proliferation has been widely studied. Although inhibitory effects of iodide are well established, it has been observed that low concentrations of iodide could stimulate thyroid cell proliferation (Pisarev 1985, Smerdely et al. 1993). Therefore, we explored whether iodide concentrations that could be generated in the total or partial deiodination of 1-0 nM T3, could exert a proliferative effect in our system. The incorporation of iodide (0-3-3-0 nM) in the T3-depleted bovine thyroid follicles did not modify the \[^{3}H\]thymidine incorporation. Values obtained (c.p.m./µgDNA; mean ± s.e.m.; n=9) from three experiments in which triplicate samples were analyzed, were: control: 1174 ± 323; T3 (1-0 nM): 2611 ± 392*; KI (0-3 nM): 1227 ± 194; KI (1-0 nM): 1087 ± 218; KI (3-0 nM): 1197 ± 132 (*P<0-01 vs control).


depleted, were treated with increasing concentrations of T3 (0-1-5-0 nM) for 24 h. Bars (c.p.m./µg DNA) represent the mean ± s.e.m. (n=9) from 3 independent experiments in which triplicate values were obtained. *P<0-01 vs control.

Figure 1 Concentration-dependent increase in \[^{3}H\]thymidine incorporation induced by T3. Bovine thyroid follicles, previously T3-depleted, were treated with increasing concentrations of T3 (0-1–5-0 nM) for 24 h. Bars (c.p.m./µg DNA) represent the mean ± s.e.m. (n=9) from 3 independent experiments in which triplicate values were obtained. *P<0-01 vs control.
from the protein extract of thyroid follicles treated with T₃ (0.1–5.0 nM) for 24 h. T₃ did not significantly modify the level of expression of the EGFR in the concentration range previously demonstrated to stimulate [³H]thymidine incorporation and total TK activity.

The influence of T₃ on EGFR–tyrosine phosphorylation was examined by Western blotting using anti-phosphotyrosine (PY20) as primary antibody. Figure 7 depicts the results obtained indicating that increasing concentrations of T₃ induced a progressive increment in the tyrosine phosphorylation of the EGFR that was significant at all the T₃ concentrations tested.

Inhibition of the T₃-induced [³H]thymidine incorporation by anti-EGF antibody

To check for possible participation of EGF in the T₃-induced DNA synthesis, we explored whether the T₃-stimulated [³H]thymidine incorporation was antagonized by an anti-EGF antibody. The antibody anti-EGF was able to significantly diminish the [³H]thymidine incorporation induced by 1-0 nM T₃ as a function of the dilutions of anti-EGF tested (Fig. 8). The antibody dilutions used were also able to attenuate the [³H]thymidine incorporation in the control cells, indicating a basal activity of the growth factor.

To corroborate the ability of thyroid follicles to respond to an exogenous EGF stimulation, the effect of human EGF on the [³H]thymidine incorporation was tested. A significant increase of [³H]thymidine incorporation was produced by treatment with human EGF for 24 h. The value of [³H]thymidine incorporation provoked by EGF did not significantly differ from that induced by 1-0 nM T₃. Values obtained (c.p.m./µgDNA; mean ± s.e.m.; n = 9) were: control, 1394 ± 143; T₃ (1·0 nM), 2674 ± 284*; EGF (100 ng/ml), 2443 ± 180* (*P<0·001 vs control).

Discussion

The presence of T₃R and diverse T₃ actions in thyrocytes from different species (Pisarev 1985, Erkenbrack & Rosenberg 1986, Pisarev et al. 1986, Nakamura & Imura 1988, Akiguchi et al. 1992, Brönnegård et al. 1994, Nagy et al. 1999) support the argument that the thyroid gland is a target for T₃. Recent findings in transgenic animals suggest that T₃R play an important role in the development and growth of the thyroid gland (Gauthier et al. 1999). Some preliminary studies evaluating the potential role of TH in the thyroid follicular cell function were limited because of the high levels of endogenous T₃ in the thyroid cell that mask the effect of exogenous T₃ (Nakamura & Imura 1988, Akiguchi et al. 1992, Bidey et al. 1999). Coincidentally, in initial experiments analysing the effect of T₃ on the thymidine incorporation in non-T₃-depleted bovine thyroid follicles we obtained highly variable results including absence of response (observations not published). Therefore, we attempted an approach in which the endogenous T₃ level was maximally reduced leaving the culture system responsive to exogenous T₃.

The effect of TH on the growth regulation in the thyrocyte is not known (Bidey et al. 1999). Our results support the notion that T₃ could act as a thyroidal growth factor since a T₃-induced increase of thymidine incorporation was demonstrated. Furthermore, in our experiments the proliferative effect of T₃ was sustained by PCNA expression, a reliable marker to test cell proliferation...
(Bravo et al. 1987, Iatropoulous & Williams 1996). The concentration–response curve of PCNA expression by T₃ was similar to that of thymidine incorporation although they diverged at 5·0 nM T₃, indicating that additional mechanisms could be involved in the response of the two proliferation indexes at high T₃ concentrations.

It is known that iodide leads either to growth inhibition or induction in thyroid tissue, depending on the type of cell and the culture system (Pisarev 1985, Smerdely et al. 1993). Since an activation of the thyroidal type I 5'-deiodinase by T₃ has been observed (Mori et al. 1996), an implication of iodide in the cellular growth response to T₃ should not be discarded. However, most of the T₃ effects on the thyroid tissue do not seem to be due to its deiodination (Pisarev 1985) although slight dehalogenation of T₃ have been reported in calf thyroid slices (Juvenal et al. 1981). In agreement, iodide concentrations within the range expected to be generated from total or partial T₃ deiodination did not modify basal thymidine incorporation in our system, supporting the idea that iodide is not involved in the T₃-induced DNA synthesis.

It is known that the effect of TH is exerted mainly by the regulation of gene expression mediated by T₃R. However, it has been demonstrated that T₃ has also non-genomic actions which are rapid and not inhibited by cycloheximide (Davis & Davis 1996, Wehling 1997, Lin et al. 1999). In our system the inhibition of the T₃-stimulated thymidine incorporation by cycloheximide indicates the possible mediation of protein synthesis. This
effect is in accordance with a genomic action and supported by the observation of the maximal proliferative effect at 1·0 nM T3, a concentration very similar to the apparent Kd of the nuclear binding sites (Akiguchi et al. 1992). However, these findings do not exclude possible non-genomic actions of T3 on signal transduction pathways.

Evidence for a close functional relationship between TH and EGF in the regulation of cellular growth has been documented in vivo as well as in cultured cells of some species (Fisher & Lakshmanan 1990, Nilsson 1995) but not in thyroid cells. Variations in EGF levels seem not to be involved in the proliferative effect of T3 in our system since no changes in the EGF expression were detected after T3 exposure. It is known that the ligand-induced TK activity of the EGFR leads to the tyrosine autophosphorylation of the receptor as a previous step in the signal transduction cascade (Chen et al. 1987). We demonstrated a stimulatory effect of T3 on the expression of EGF–tyrosine phosphorylation thus evidencing an activation of the EGFR. The T3-dependent increase in the EGFR–tyrosine phosphorylation paralleled the increase in total TK activity and DNA synthesis although the persistence of high levels of tyrosine-phosphorylated EGFR was not correlated with the lack of increase in the total TK activity and thymidine incorporation at 5·0 nM T3. This may indicate the involvement of some other factors in the diminution of TK activity at this T3 concentration. In accordance, several reports demonstrated that the EGFR activation also resulted in the phosphorylation of EGFR on threonine residues, leading to receptor internalization or TK activity inhibition (Sibley et al. 1988). This biphasic T3 effect on DNA synthesis may also be a consequence of the T3 deiodination (Mori et al. 1996) or the phosphorylation of multiple proteins including the T3R, an event reported to play a role in regulating the action of T3R as well as other nuclear hormone receptors (Sugawara et al. 1994, Solomon et al. 1999).

**Table 2** Effect of T3 on tyrosine kinase (TK) activity

<table>
<thead>
<tr>
<th>T3 (nM)</th>
<th>Tyrosine kinase activity (pmol P/30 min)</th>
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<tbody>
<tr>
<td>0 (Control)</td>
<td>2·16 ± 0·48</td>
</tr>
<tr>
<td>0·1</td>
<td>2·72 ± 0·16</td>
</tr>
<tr>
<td>0·5</td>
<td>4·12 ± 0·68*</td>
</tr>
<tr>
<td>1·0</td>
<td>4·36 ± 0·68*</td>
</tr>
<tr>
<td>5·0</td>
<td>2·98 ± 0·79</td>
</tr>
</tbody>
</table>

Thyroid follicles in primary culture previously T3 depleted were treated with T3 (0·1–5·0 nM) for 24 h. Total tyrosine kinase activity was determined in cellular protein extracts as indicated in Materials and Methods. Each point (pmol P/30 min) represents the mean ± S.E.M. (n=6) from 3 individual experiments in which duplicate values were obtained. *P<0·001 vs control.

cancer cell lines (Yin et al. 1999). The involvement of TK activity in the proliferative effect of T3 was further confirmed by the T3-induced increase of the total TK activity. The abolishment of the T3-induced DNA synthesis by tyrphostin A25 was in favor of an EGFR-mediated TK activity in the T3-stimulated proliferation. This hypothesis was further confirmed by the inhibition of the T3-induced TK activity in the presence of an anti-EGFR antibody.

Changes in the EGFR expression by TH have been documented in vivo as well as in cultured cells of some species (Fisher & Lakshmanan 1990, Nilsson 1995) but not in thyroid cells. Variations in EGF levels seem not to be involved in the proliferative effect of T3 in our system since no changes in the EGF expression were detected after T3 exposure. It is known that the ligand-induced TK activity of the EGFR leads to the tyrosine autophosphorylation of the receptor as a previous step in the signal transduction cascade (Chen et al. 1987). We demonstrated a stimulatory effect of T3 on the expression of EGF–tyrosine phosphorylation thus evidencing an activation of the EGFR. The T3-dependent increase in the EGFR–tyrosine phosphorylation paralleled the increase in total TK activity and DNA synthesis although the persistence of high levels of tyrosine-phosphorylated EGFR was not correlated with the lack of increase in the total TK activity and thymidine incorporation at 5·0 nM T3. This may indicate the involvement of some other factors in the diminution of TK activity at this T3 concentration. In accordance, several reports demonstrated that the EGFR activation also resulted in the phosphorylation of EGFR on threonine residues, leading to receptor internalization or TK activity inhibition (Sibley et al. 1988). This biphasic T3 effect on DNA synthesis may also be a consequence of the T3 deiodination (Mori et al. 1996) or the phosphorylation of multiple proteins including the T3R, an event reported to play a role in regulating the action of T3R as well as other nuclear hormone receptors (Sugawara et al. 1994, Solomon et al. 1999).
A TH-dependent increase in the level of EGF mRNA and immunoreactive EGF in the mouse thyroid tissue has been reported (Osawa et al. 1991, Sheflin et al. 1993). Our findings support the notion that an EGF/EGFR activation could mediate the proliferative response to T3 by the demonstration of a T3-induced DNA synthesis along with an EGFR-associated TK activity. The inhibition of the T3-induced thymidine incorporation by an anti-EGF antibody favors this hypothesis.

Previous evidence indicates that T3 exerts mainly inhibitory effects on several thyrotropin (TSH)-stimulated functional parameters in the thyroid (Pisarev 1985). Recently, in FRTL-5 cells it was observed that T3 attenuated the expression of the sodium iodide symporter (Spitzweg et al. 1999). By coincidence, EGF is a strong inhibitor of thyroid function in several species, as well as a potent proliferative agent (Fisher & Lakshmanan 1990, Nilsson 1995). Therefore, the possibility that the inhibitory effect of T3 on thyroid function could be mediated by an autocrine action of EGF as we propose for the T3-proliferative effect should be addressed.

It is widely accepted that the administration of TH inhibits thyroid function and growth by suppressing TSH release (Utiger 1995). We believe that the contribution of TH on the thyroid gland proliferation should be considered as an additional regulatory pathway. It is possible to speculate that TH participates in a short-loop feedback mechanism in concert with EGF to modulate thyroid function. Thus, in the thyocyte T3 might induce the biosynthesis and release of EGF and in turn, EGF could diminish the biosynthesis of TH. The T3 proliferative action on the thyroid cell seems to operate better at T3 concentrations closer to the affinity constant value of the T3R and poorly at high T3 levels. This could be regarded as a protective mechanism of the thyroid cell against the exposition to large amounts of TH. A lack of control of this mechanism could result in unregulated thyroid cell growth. Hence, a T3-induced proliferation could be involved in the goiter induction that occurs in patients with mild TH deficiency (Utiger 1995) as well as in the TH resistance syndrome (Weiss & Reffetof 1997). In these entities the relatively insufficient T3 activity could render the thyroid cell more sensitive to the T3 proliferative effect.

Taken together, these results indicate that T3 is a potential proliferative agent in the thyroid cell by a mechanism that involves, at least in part, an EGFR-mediated TK activation by the enhanced expression, availability or actions of autocrine EGF. The physiological and clinical relevance of the relationship between T3 and EGF in the thyroid tissue remains to be determined.
Acknowledgements

We would like to thank Drs G Chiabrando, G Bonacci, J Daniotti and A Zurita for assistance in Western blot assays, Dr G Disserio for T₃ measurement, Drs H Maccioni and C Argarana for helpful suggestions, and Paola Safarsi for language support. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT; FONCYT), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SeCyT-UNC) and Consejo de Investigaciones Científicas y Tecnológicas de la Provincia de Córdoba (CONICOR). This work has been presented in part at the VIII Congress of the Latin-American Thyroid Society, May 1999. Foz do Iguazu, Brazil (Abstract 4). MDF and CGP are fellows from SeCyT-UNC and CONICET, respectively. AHC is a research member of CONICET.

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Received 8 December 1999
Accepted 7 March 2000

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