Decreased expression of retinoid nuclear receptor (RARα and RARγ) mRNA determined by real-time quantitative RT-PCR in peripheral blood mononuclear cells of hypothyroid patients

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Abstract

In vivo assessment of the cellular impact of thyroid hormones on target tissues might be of help for physiological studies and to evaluate the consequences of various diseases of the thyroid gland in humans. Given the tenuous relationship between retinoid and tri-iodothyronine (T3) status and that retinoids have also intracellular roles, the aim of this study was to determine the effect of hypothyroidism on the expression of T3 nuclear receptors (TR) and retinoic acid nuclear receptors (RAR, RXR) in human peripheral blood mononuclear cells (PBMC). Using real time RT-PCR, we quantified the relative amount of mRNA of the thyroid (TRβ1 and TRβ2) and retinoid (RARα, RARγ, and RXRα) nuclear receptors in PBMC of euthyroid (n=22) compared with hypothyroid (n=22) subjects. Classical plasma parameters (free T3 (FT3), free thyroxine (T4) (FT4), thyroid-stimulating hormone (TSH), retinol (ROH), retinol-binding protein (RBP) and transthyretin (TTR)) were also measured. In hypothyroid subjects, the concentration of TSH was elevated, and dramatically low T3 and T4 concentrations were associated with a decrease in the expression of TRβ1. Expression of RARα and RARγ significantly decreased in hypothyroid versus control subjects, while an increased concentration of ROH was emphasised by hypothyroidism. These results first indicated that primary hypothyroidism induces hypoactivation of the retinoid nuclear pathway in PBMC, which was not predicted by the plasma ROH level. Further investigations will be necessary to evaluate these parameters in very small changes in thyroid hormone production such as mild (subclinical) hypothyroidism.

Introduction

The physiological actions of thyroid hormones in the regulation of diverse cellular activities, including normal growth and general metabolism, are well defined (reviewed by Yen 2001). Thyroid disorders such as hyper- and hypothyroidism induce considerable consequences in children and adults. For example, children born with congenital hypothyroidism who lack thyroid hormones during a circumscribed period of early development are at risk of brain damage and mental retardation (reviewed by Rovet & Daneman 2003). Assessment of thyroid function in humans is commonly performed with plasma free thyroxine (T4) and thyroid-stimulating hormone (TSH) measurements. Low serum free (F)T4 contrasting with elevated TSH concentrations confirms the diagnosis of hypothyroidism and signifies that it is due to failure of the thyroid gland (primary hypothyroidism). Thus, in human studies, only plasma parameters are used to characterise the thyroid hormone status of an individual even though thyroid hormones have a mainly intracellular role. Indeed, there is strong evidence pointing to the nucleus as the principal site for the initiation of thyroid hormone action (Oppenheimer 1999). Assessment of the cellular impact of thyroid hormone deficiency might be of help in circumstances such as thyroid hormone resistance. Elsewhere, many older patient have abnormal TSH levels without other alterations in serum thyroid hormone levels, a condition defined as subclinical hypothyroidism that occurs in 4–8% of elderly subjects (Surks et al. 2004). The clinical consequences of this condition are controversial; therefore, there is need for the development of alternative markers of the cellular
impact of thyroid hormones. Within the cell, these actions are mediated by specific triiodothyronine (T₃) nuclear receptors (TRα and TRβ), which regulate the expression of targeted genes (reviewed by Yen 2001, Viguerie & Langin 2003). These receptors belong to the superfamily of hormone nuclear receptors, which function as ligand-activated transcription factors (Aranda & Pascual 2001).

Like TR, retinoid nuclear receptors (RARα, RARβ and RARγ and RXRα, RXRβ and RXRγ) are members of the hormone receptor superfamily and regulate target genes in response to RA (retinoic acid) ligand (RA is the active form of vitamin A) (Marill et al. 2003). Vitamin A, via RA, exerts a wide variety of profound effects on growth, tissue differentiation and homeostasis (Sporn et al. 1994, reviewed by Bastien & Rochette-Egly 2004). It has been shown that RXR forms heterodimers with either RAR or TR in order to regulate gene transcription by interacting with distinct sequences in the promoter of target genes. The fact that RXR is the essential common partner for the functional heterodimer indicates that thyroid hormones and retinoid signalling pathways are closely related (Schräder & Carlberg 1994, Mangelsdorf & Evans 1995, Li et al. 2002). We have shown that a decrease in maximal binding capacity of TR and RAR in the liver, as well as decreased affinity for their ligand, has been observed in hypothyroid rats compared with controls (Pallet et al. 1994). Inversely, vitamin A status may influence thyroid hormone signalling. Indeed, decreased expression of TR has been observed in the liver and the brain of rats fed a vitamin A-deficient diet (Higuere et al. 1989, Husson et al. 2003). Likewise, many interactions between thyroid hormones and retinoid nuclear signalling have been reported, at the plasmatic level, in studies performed in humans or animals. Hypothyroidism is associated with elevated retinol (ROH) and β-carotene concentrations in humans (Goswami & Choudhury 1999). Mice lacking the transthyretin gene (TTR), a protein that binds and carries thyroxin, exhibit decreased ROH and retinol-binding protein (RBP) concentrations (van Bennekum et al. 2001). Moreover, Coya et al. (1997) have reported that TSH secretion in rats is partly regulated in vivo by RA.

Expression of TRα and TRβ in peripheral blood mononuclear cells (PBMC) is sensitive to thyroid status (Meier-Heusler et al. 1995). The RA nuclear receptors RARα, RARγ, RXRα and RXRβ have also been found in these cells (Szabova et al. 2003). Given that retinoids and thyroid hormones have mainly intracellular action, the aim of the present study was twofold: firstly, to establish the effect of primary hypothyroidism on the expression of TRα and TRβ in human PBMC; secondly, to clarify the effect on the expression of the retinoid nuclear receptors RARα, RARγ and RXRα in PBMC. The amount of mRNA of TRα, TRβ, RARα, RARγ and RXRα was quantified by the real-time RT-PCR method.

The classical thyroid parameters of euthyroid and hypothyroid subjects were also measured (FT₃, FT₄ and TSH), as well as those classically used to qualify the vitamin A status (ROH and RBP concentrations and the RBP:TTR ratio assessment).

Materials and methods

Subjects

Forty-four men and women volunteers are recruited for this study: 22 (24–57 years old) in the euthyroid group and 22 (24–67 years old) in the hypothyroid group. Subjects of the euthyroid group were selected from volunteers, according to their health (and thyroid status). Hypothyroid patients were recruited in the Endocrinology, Diabetology, and Metabolic Disease Unit of l'Hôpital Haut-Levêque, a university teaching hospital in Bordeaux, France. All patients underwent total thyroidectomy for papillary carcinoma and were referred for complementary 131I therapy. For this purpose, thyroid hormone replacement therapy was withdrawn for 4 weeks, and all patients were on a mild low-iodine diet during these 4 weeks. Blood samples were collected immediately prior to radioactive iodine therapy. All patients gave informed consent.

Health status

Venous blood was collected from all subjects after overnight fasting (≥ 12 h after previous meal). Diagnostics kits (RIA and IRMA; Immunootech, Marseille, France) were used to measure plasma FT₃ (n = 22 in euthyroid group), FT₄ (n = 22 in euthyroid group) and TSH (n = 20 and n = 22 in euthyroid and hypothyroid groups respectively) concentration. The plasma ROH (n = 20 and n = 22 in euthyroid and hypothyroid groups respectively) concentration was determined by HPLC by the method of Leclercq and Bourgeay-Causse (1981). Plasma TTR (n = 19 and n = 22 in euthyroid and hypothyroid groups respectively) and RBP (n = 19 and n = 22 in euthyroid and hypothyroid groups respectively) concentrations were measured by the immunonephelometric process (Nephelometer Analyser II; Behring Diagnostics, Deerfield, IL, USA). Total plasma cholesterol (TC) (n = 21 and n = 22 in euthyroid and hypothyroid groups respectively) and total triglyceride (TTG) (n = 21 and n = 22 in euthyroid and hypothyroid groups respectively) concentrations were measured by the enzymatic method with a Synchron CX5 analyser (Beckman Coulter, Villepinte, France).

Preparation of PBMC

PBMC were isolated by density gradient centrifugation with Ficoll-Paque Plus solution (Amersham Biosciences,
F-91898 Orsay Cedex, France). After overnight fasting, venous blood (5 ml) was drawn into an EDTA-coated vacutainer tube, layered onto 4 ml Ficoll-Paque Plus solution and then centrifuged at 400 g for 20 min at 20 °C. PBMC were removed from the plasma-Ficoll interface and washed twice in 1 M PBS, pH 7·2, to remove platelets, Ficoll-Paque and plasma. PBMC were then suspended in TRIzol Reagent (Invitrogen, Cergy-Pontoise, France) for total RNA preparation. PBMC were homogenised in 1 ml TRIzol containing oligonucleotide primers (5 µM of each), MgCl₂ (5 mM) and DNA Master SYBR green (Roche) containing Taq DNA polymerase, reaction buffer, dNTP and the double-stranded DNA-specific fluorescent dye, SYBR green I. Amplification occurred in a two-step procedure: denaturation at 95 °C for 10 min and 30 cycles with denaturation at 95 °C for 8 s, annealing at 66 °C for 6 s and extension at 72 °C for 10 s. Acquisition of the fluorescent signal from the samples was carried out at the end of elongation step. The forward and reverse primer sequences are shown in Table 1. Specificity of primers was validated through the verification of RT-PCR product specificity. The identity of amplified products was verified by sequencing with the Dye Terminator Reaction Cycle Kit (Perkin-Elmer, Norwalk, CT, USA) and analysed on an ABI PRISM 377 automated DNA sequencer (Perkin-Elmer).

Quantification data were analysed by LightCycler analysis software, Version 3·5 (Roche). In this analysis, the background fluorescence was removed by setting a noise band. The log-linear portion of the standard amplification curve was identified, and the crossing point (Cp) was the intersection of the best-fit line through the log-linear region and the noise band. The log-linear region of the standard amplification curve was a plot of the Cp versus the amount of the initial cDNA used for amplification. The Cp was always

Table 1 Primers used for LightCycler real-time PCR

| PCR primer pair | Ref                  | Sequence | Product length (bp) | Position
|-----------------|---------------------|----------|---------------------|-----------
| PBGD            | Raich et al. 1986   | F: 5′-TGCAAGCATCAGGAGACCTCTTG-3′ | 743–763 | 90
|                 |                     | R: 5′-GCACGGTACTGGCACAC-3′       | 812–832 | 280–304 | 165
| Cyclophilin     | Haendler et al. 1987| F: 5′-TCCTAAAGCATAGGTCCTGGCAT-3′| 421–445 | 235
|                 |                     | R: 5′-CGCTCCATGGGCTCCACAATATTCA-3′| 519–538 | 1040–1064 | 142
| RARα            | Giguere et al. 1987 | F: 5′-CTGCCAGTACTGGCATGTC-3′    | 837–856 | 228
|                 |                     | R: 5′-GTTCTAGATGACTCGAAGGCGG-3′  | 1075–1098 | 119
| RARγ            | Krust et al. 1989   | F: 5′-CCTGCCAGTACTGGCATGTC-3′   | 861–882 | 142
|                 |                     | R: 5′-GAGCCACCTCAGACATTTGC-3′   | 981–1002 | 119
| RXRα            | Mangelsdorf et al. 1990| F: 5′-CGACCCTGTCACCAAATTTGC-3′ | 838–860 | 119
|                 |                     | R: 5′-GAGCCACCTCAGACATTTGC-3′   | 981–1002 | 119
| TRα             | Laudet et al. 1991  | F: 5′-GTTCCTAGATGACTCGAAGGCGG-3′| 935–956 | 90
|                 |                     | R: 5′-CCTGCAGTTGAGGCTCTGGTCG-3′ | 334–358 | 90
| TRβ             | Weinberger et al. 1986| F: 5′-CGGAAGCTAGTCCAGAGCCAGAAAC-3′ | 399–423 | 90

F 82–83
R 82–83
F 280–304
R 280–304
F 519–538
R 519–538
F 837–856
R 837–856
F 1040–1064
R 1040–1064
F 861–882
R 861–882
F 981–1002
R 981–1002
F 838–860
R 838–860
F 935–956
R 935–956
F 334–358
R 334–358
F 399–423
R 399–423
the same for a given dilution of cyclophilin or porphobilinogen deaminase (PBGD) cDNA, whatever the thyroid status of the subjects, demonstrating that cyclophilin and PBGD mRNA expression were not altered by thyroid status and could be used as normaliser for data from target mRNA (Fig. 1). The relation between the Cp and the initial amount of cDNA was found to be linear. The correlation coefficient (r) was 1, and PCR amplification efficiencies of the target and the housekeeping genes were similar and close to 100% (Fig. 1). These standard curves were used to estimate the concentration of both the target and the housekeeping genes in each sample. The results were then normalised by the ratio of the relative concentration of target to that of housekeeping in the same sample.

Cyclophilin cDNA was used as housekeeping for the relative quantification of cDNA of RARα (n=15 and n=22 in euthyroid and hypothyroid groups respectively), RXRα (n=12 and n=20 in euthyroid and hypothyroid groups respectively) and TRα (n=19 and n=20 in euthyroid and hypothyroid groups respectively), and PBGD was used for the relative quantification of RARγ (n=16 and n=20 in euthyroid and hypothyroid groups respectively) and TRβ (n=14 and n=19 in euthyroid and hypothyroid groups respectively).

**Statistical analysis**

Data are expressed as means ± S.E.M. All statistics were analysed by Statgraphics Plus software. The statistical significance of differences between means was calculated by ANOVA followed by Student’s t-test, in which equality of S.D. was not assumed because it appeared different between the two groups. Linear regression was used to analyse the relationships between variables. Statistical significance was accepted at P<0.05.

**Results**

**Thyroid status and mRNA expression of TRα and TRβ in PBMC from euthyroid and hypothyroid subjects**

The results are summarised in Tables 2 and 3.

The plasma free thyroid hormone concentrations (FT3 and FT4) and the TSH concentration are used clinically to diagnose thyroid disorders. In the hypothyroid group, the FT3 and FT4 concentrations were undetectable, while the TSH concentration was dramatically increased compared with the euthyroid group (P<0.0001).

*Figure 1* Standard curves for housekeeping genes: (A) cyclophilin, ● in euthyroid group and ○ in hypothyroid group; (B) porphobilinogen deaminase (PBGD), ● in euthyroid group and □ in hypothyroid group. Standard curves for target genes in euthyroid group: (C) RXRα (○), RARα (▲) and TRα (+) relative to cyclophilin (●); (D) RARγ (△) and TRβ (*) relative to PBGD (●).
The mRNA expression of the thyroid nuclear receptors was assessed in PBMC of healthy euthyroid versus hypothyroid subjects. The amount of TRα mRNA was significantly reduced in the hypothyroid compared with the euthyroid group (–25%, \( P=0.016 \)). On the other hand, the amount of TRβ mRNA remained unchanged (\( P=0.18 \)).

Vitamin A status and mRNA expression of RARα, RARγ and RXRα in PBMC from euthyroid and hypothyroid subjects

The results are summarised in Tables 2 and 3.

The vitamin A status of all subjects was determined by measuring ROH and RBP concentrations. RBP was proposed as a simple surrogate for vitamin A assessment. Hypothyroid subjects exhibited a significant increase in concentration of ROH (+26%, \( P=0.005 \)) and no change in RBP concentration. As a consequence, the ratio ROH:RBP also increased in the hypothyroid compared with the euthyroid group (+24%, \( P<0.001 \)). The molar ratio RBP:TTR, used as an indirect method of vitamin A assessment, was unchanged in the hypothyroid group compared with the control. TTR, which is both a protein that binds and carries T4 and a marker of malnutrition, was unaffected by hypothyroidism. Several subtypes of retinoid receptors were expressed in human PBMC. The relative abundance of RARα and RARγ mRNA was significantly reduced in PBMC of hypothyroid compared with euthyroid subjects (–18%, \( P=0.016 \); –25%, \( P=0.002 \), respectively), whereas the amount of mRNA encoding the RXRα receptor isoform was not modified by hypothyroidism in the PBMC.

Lipid parameters

The total cholesterol and fasting triglyceride concentrations increased in hypothyroid compared with euthyroid subjects (+64%, \( P<0.01 \); +132%, \( P<0.01 \), respectively) (Table 2).
Table 3 Effect of hypothyroidism on the relative expression of T₃ and retinoic acid nuclear receptors in human PBMC

<table>
<thead>
<tr>
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<th>Euthyroid group (total n=22)</th>
<th>Hypothyroid group (total n=22)</th>
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<tbody>
<tr>
<td></td>
<td>Mean± S.E.M.</td>
<td>Range</td>
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<tr>
<td>RARα (% cyclophilin)</td>
<td>35.9±2.1</td>
<td>22–46</td>
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<tr>
<td>(15)</td>
<td></td>
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<tr>
<td>RARγ (% PBGD)</td>
<td>193.1±12.7</td>
<td>111–283</td>
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<tr>
<td>(16)</td>
<td></td>
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<tr>
<td>RXRα (% cyclophilin)</td>
<td>25.2±1.1</td>
<td>20–30</td>
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<tr>
<td>(12)</td>
<td></td>
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<tr>
<td>TRα (% cyclophilin)</td>
<td>4.9±0.2</td>
<td>3.5–6.8</td>
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<tr>
<td>(19)</td>
<td></td>
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<tr>
<td>TRβ (% PBGD)</td>
<td>12.6±0.6</td>
<td>9.1–16.2</td>
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<tr>
<td>(14)</td>
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Statistical analyses were performed by ANOVA followed by Student’s t-test with inequality of s.d.
*Significantly different from euthyroid group, P<0.05.

Discussion

Hypothyroidism, a common thyroid disorder, affects every major organ system and metabolic process (Heitman & Irizarry 1995). The clinical manifestations of overt hypothyroidism and the effects of this dysfunction on other biological markers, such as lipid status, are well defined at the circulation level in humans. In the present study, total cholesterol and triglycerides were assessed in euthyroid and hypothyroid groups. A classical hypothyroid-related increase in total cholesterol and triglyceride concentrations was described that could be directly attributed to the collapse of the thyroid status because the regulation of the lipid metabolism is T₃ dependent (Gullberg et al. 2000, Macchia et al. 2001). Indeed, Efstathiadiou et al. (2001) and Duntas L H (2002) have reported that the composition and transport of lipoproteins are slightly or seriously disturbed in thyroid diseases.

Patients recruited for this study were thyroidectomised for thyroid carcinoma prior to the study, and thyroid hormone replacement therapy was withdrawn for 4 weeks in order to achieve complementary ¹³¹I therapy. As expected, patients exhibited a massive rise in TSH concentration with an almost complete disappearance of T₃ and T₄ from the plasma. These results are consistent with profound although recent hypothyroidism.

The amounts of TRα and TRβ mRNA were quantified in order to determine the nuclear function of thyroid hormones in the PBMC. Hypothyroidism led to significantly decreased expression of TRβ mRNA in PBMC, contrary to TRα mRNA expression, which was not significantly changed.

Hormonal regulation of TR in human lymphocytes has been demonstrated, but the present results differ from previous observations. Indeed, TRα mRNA has been found to be upregulated by hypothyroidism in lymphocytes (Nagayama et al. 1988). Similarly, Li et al. (1990) have described an increase in the maximal binding capacity of TR in hypothyroid conditions. Meier-Heusler et al. (1995), in developing an assay for the quantitative determination of TRβ mRNA levels in human tissue samples, have found no change in TRβ expression in blood cells from hypothyroid subjects. The present result differed from this last observation, because only a slight but not significantly increased of TRα mRNA expression was observed. The divergence in TRα and TRβ expression between the present results and those of the studies previously mentioned may be due to the sensitivity of the methods used. Indeed, the Northern blot analysis, dot-blot hybridisation and quantitative competitive PCR techniques previously applied are less specific and sensitive than the real-time RT-PCR performed in this study.

In the euthyroid group, the level of expression of TRβ appeared to be well correlated to the FT₃ concentration (r=0.60, P=0.022). In the hypothyroid group, such associations were lost in that T₃ disappeared from the circulation.

The effects of hypothyroidism on retinoid nuclear receptors are poorly defined in humans. The circulating levels of the major dietary retinoid detectable in blood, ROH, and its carrier in blood, RBP, have been determined (Almekinder et al. 2000, de Pee & Dary 2002). This study established that an increase in plasma ROH level is associated with hypothyroidism. A comparable result has already been obtained in hypothyroid women, who also exhibited an increase in β-carotene in plasma (Goswami & Choudhury 1999). The RBP concentration appeared not to be affected by hypothyroidism; indeed, it remained within the laboratory reference range in hypothyroid patients.
Consequently, the ROH:RBP ratio, which is normally around 1 (revealing the 1:1 molar complex between ROH and RBP) increased with hypothyroidism. Moreover, it is interesting to note that no subject had a fasting plasma ROH concentration under 1.4 µmol/l, a threshold assumed to indicate a risk of vitamin A deficiency (Hercberg et al. 1994).

TTR plasma concentration is also a sensitive indicator of malnutrition and illness (Robbins 2002). In the present case, hypothyroidism appeared to be without effect on this concentration in plasma. This finding, which has been previously observed by Marrocco et al. (1984), was a further indicator allowing us to say that the population was not affected by malnutrition, particularly lack of iodine. Indeed, a reduction of TTR production rate in combination with its very rapid rate of disappearance from the circulation has been found to occur with malnutrition (Ingenbleek et al. 1975). Moreover, Centanni et al. (1995, 1998) found that the RBP and the TTR concentrations decreased in children and adults exposed to mild or overt hypothyroidism due to iodine deficiency. The discrepancy between their

Figure 2 Correlations between (A) TRβ mRNA levels in PBMC and FT3 concentrations in the euthyroid group (♦: n=14; r=0.60, P=0.022) and (B) RARγ mRNA levels in PBMC and retinol concentration in the euthyroid (♦: n=15; r=0.52, P=0.045) and hypothyroid groups (♦: n=20; r=0.15, P=0.53).
Hypothyroidism did not induce any modifications of plasma thyroid hormones and TSH concentrations but also a decreased expression of TRβ in PBMC. Interestingly, it seems that thyroidectomy-induced hypothyroidism also generates modifications in the cellular bioavailability of retinoic acid, the manifestation of which is, at the nuclear level, the downregulation of some retinoic acid receptors in human PBMC. From this perspective it might be speculated that hypoactivation of retinoid signalling occurs in other tissues of the hypothyroid individual, similarly to our findings in the liver of hypothyroid rats. (Pallet et al. 1994, Coustaut et al. 1996).

Elsewhere, measurement of retinoid and thyroid receptor expression might be a useful tool for the cellular assessment of the impact of thyroid hormones. Further studies conducted in so-called subclinical situations such as hypothyroidism will be useful to determine the true impact of subtype hormone deficiency at the cellular level and the usefulness of these peripheral cellular markers to characterise the hormonal status when classical thyroid hormone measurement displays only minor changes.

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