The biological relevance of thyroid hormone receptors in immortalized human umbilical vein endothelial cells

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Abstract

The gene expression of thyroid hormone receptors (TR) in ECRF24 immortalized human umbilical vein endothelial cells (HUVECs) was investigated at both the mRNA and the protein level. Endothelin-1 (ET-1) and von Willebrand factor (vWF) production were measured in cell supernatants with a two-site immunoenzymatic assay. Scatchard analysis yielded a maximum binding capacity of 55 fmol T3/mg DNA (± 200 sites/cell) with a $K_d$ of 125 pmol/l. Messenger RNAs encoding for the TRα1 and the TRα2 and the TRβ1 were observed. The approximate number of mRNA molecules per cell was at least 50 molecules per cell for TRα1, five for TRα2 and two for TRβ1. Immunocytochemistry revealed (peri)nuclear staining for TRβ1, TRα1 and TRα2. ET-1 and vWF secretion did not increase upon addition of T3 ($10^{-10}$–$10^{-6}$ M). Immortalized ECRF24 HUVECs express TR, but at low levels. The number of TRs per endothelial cell is probably too low to be functional and no change in ET-1 or vWF production was found after addition of T3. Therefore we conclude that the genomic effects of T3 are unlikely to occur in these immortalized HUVECs.


Introduction

Endothelial cells appear to function as a large endocrine organ in the human body (Vane et al. 1990). Little is known about the interaction of thyroid hormones with endothelial cells. This is of potential interest because endothelial cell dysfunction is involved in cardiovascular disease (Cines et al. 1998) and thyroid dysfunction is a frequently occurring condition with an increased risk for cardiovascular complications (Polikar et al. 1993). It would therefore be of interest to have a model system in which to study the interaction of thyroid hormone with endothelial cells. The main action of thyroid hormone is thought to be mediated by affecting gene expression by binding of triiodothyronine (T3) to its nuclear thyroid hormone receptor (TR), which in turn binds to a specific DNA element regulating transcription (Lazar 1993). At least four isoforms of the TR are known, namely α1, α2, β1 and β2, which are present in a splicing variant of the α gene that is not able to bind T3 but may act in a dominant-negative fashion on T3-dependent gene expression. TRα1 and TRβ1 usually act in a positive fashion.

A few studies, in which endothelial cells from various sources were studied with different probes and techniques, have reported on the presence of TRs in these cells and on postreceptor effects. Immunofluorescence studies, using polyclonal antibodies raised in mice against bovine TRs, showed both cytoplasmic and perinuclear staining in cultured bovine aortic cells, sinusoids, liver tissue and intima of large vessels in spleen (Sellitti et al. 1985). Also in bovine aortic endothelial cells mRNAs for TRα1, TRα2 and TRβ1 were found using Northern blot and S1 nuclease protection. At the protein level, specific binding with $^{125}$I[T3] was measured in these bovine cells yielding a $K_d$ of 200 pmol/l and a $B_{max}$ of 5·6 pmol/mg protein (Hu et al. 1994). The human endothelial cell line ECV304 expresses mRNAs for TRα1, TRα2 and TRβ1 as measured by Northern blot and in situ hybridization analysis (Dietrich et al. 1997). In these cells increased intercellular adhesion molecule-1 mRNA expression was observed within hours after stimulation with T3. In
cultured primary human umbilical vein endothelial cells (HUVECs) (peri)nuclear and cytoplasmic staining of TRβ1 was found as well as increased expression of both mRNA and protein for endothelin-1 (ET-1), von Willebrand factor (vWF) and fibronectin after 13 days stimulation with T_3 (Baumgartner-Parzer et al. 1997). Recently, Lansink et al. (1998) studied human microvascular endothelial cells derived from penile foreskin with an RT-PCR technique (using common primers for both TRα1 and TRα2) and found TRα and TRβ1 mRNA expression.

To explore the possibility of using the immortalized HUVEC line ECRF24 as a model for the interaction of thyroid hormone and endothelium, we studied TR isoform expression in these cells in a more quantitative manner using a real-time PCR technique. In addition, we performed Scatchard analysis, Western blotting and immunocytostemcy to study TR isoform protein expression and looked for T_3-induced ET-1 and vWF expression (postreceptor effect).

Materials and Methods

Materials

RPMI 1640 (with Hapes and glutamine), Medium 199 (M199) (with Na_2HCO_3 and glutamine), human serum, 1-glutamine, antibiotics/antimycotics (penicillin–streptomycin–fungizone) and trypsin/versene (EDTA) were purchased from Bio Whittaker, Walkersville, MD, USA. Geneticin sulphate (G418) was obtained from Gibco, Paisley, Scotland. 3,5,3'-Triidothyronine (T_3) was obtained from Henning GmbH, Berlin, Germany, [125I]T_3 (specific activity 2200 Ci/mmol) from New England Nuclear, Boston, MA, USA. Culture flasks were from Costar Corporation, Cambridge, MA, USA. Gelatin (from swine skin type 1) was from Sigma, St Louis, MO, USA.

Polyclonal antisera, directed against synthetic TR-isoform-specific (α1, α2 and β1) peptides, coupled to keyhole limpet haemocyanin, were raised by immunization of New Zealand White rabbits (Eurogentec, Seraing, Belgium). Specificity of the antisera was tested by preimmune staining, preadsorption of the antisera with homologous antigen and by Western blots (C L Vuyts, U A Unmehopa, J M Bisselink, A A Sluiter, O Bakker, W M Wiersinga, D F Swaab & E Fliers, unpublished observations).

Cell culture

HUVECs (ECRF24), immortalized using an amphotrophic, replication-deficient retrovirus containing the E6/E7 genes of human papilloma virus (a kind gift of Dr R Fontijn, Department of Biochemistry, Academic Medical Centre, Amsterdam) (Fontijn et al. 1995), were cultured in 75 cm² flasks coated with 1% gelatin and maintained at 37 °C in 5% CO_2. Culture medium consisted of equal volumes of RPMI 1640 (with Hapes and glutamine) and M199 (with Na_2HCO_3 and glutamine) containing 20% (v/v) heat-inactivated human serum, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0·25 µg/ml) and G418 (100 µg/ml). Confluent cells were split after treatment with trypsin (500 mg/l)/EDTA (200 mg/l). Cell viability was assessed by trypan blue exclusion.

The levels of free T_3 were measured in the culture medium using a Delfia free T_3 time-resolved fluoroimmunoassay (Wallac, Perkin-Elmer, Finland).

Assay of TR mRNA

Confluent ECRF24 cells were grown in six-well plates. Total cellular RNA was isolated using Tri-Pure reagent (Roche Molecular Biochemicals, Mannheim, Germany) which was then reverse transcribed using random primers and a first-strand cDNA synthesis kit (AMV) (Roche Molecular Biochemicals). Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals) in a 20 µl volume. Detection was in the SYBR Green I format using the LightCycler FastStart DNA master SYBR Green I kit with the following program: 10 min 95°C to activate the polymerase followed by 45 cycles of 0 s 95°C, 5 s 52°C, 10 s 72°C. Products were identified by their melting curve and by gel electrophoresis. Primer sequences and standards were as described (Bakker et al. 1998). Standards were added in the range of 1000 to 0·1 attograms (ag)/20 µl. Standard curves were linear over this range and in all three cases had an r²=1·00. The amount of isoform-specific mRNA molecules per cell was estimated taking into account a cDNA synthesis efficiency of about 30% and an average value of 20 pg total RNA per cell.

Assay of TR protein

Scatchard analysis Isolation of endothelial cell nuclei was performed by twice washing confluent ECRF24 cells with ice-cold PBS. The adherent cells were removed by scraping with a rubber policeman. Cells were taken in solution A (Tris 20 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, glycerol 5% (v/v), sucrose 0·25 M, pH 7·6) and centrifuged at 500 g. The cell pellet was lysed in solution A containing 0·5% Triton X-100, again centrifuged at 500 g and subsequently homogenized in solution A. Nuclei were harvested after centrifugation at 500 g and suspended into solution B (Tris 20 mM, glycerol 5%, sucrose 0·25 M, EDTA 1 mM, NaCl 50 mM, pH 7·6).
Freshly prepared nuclear suspension (0.25 to 0.1 ml) was incubated in solution B with 11–15 fmol $[^{125}I]T_3$ in the presence of 5 mM dithiothreitol for 2 h at 22 °C in a shaking water bath. Total volume was 0.5 ml, and all incubations were done in triplicate. The incubation was stopped by chilling the samples on ice; thereafter the nuclei were pelleted (4 °C, 500 g) and washed twice with solution B containing 0.5% Triton X-100. Specific binding was calculated by subtracting the radioactivity remaining with the nuclear pellet of parallel incubations containing an excess (10$^{-6}$ M) of non-radioactive T3. To determine the association constant ($K_a$) and maximum binding capacity (MBC) of the binding of T3 to the nuclear receptors, Scatchard analysis was performed. To this end increasing amounts of non-radioactive T3 were added to the test tubes. Using the DNA-binding fluorochrome Hoechst 33258 the amount of DNA per test tube was measured (Gallagher 1995). The number of binding sites per cell was calculated assuming one T3 binding site per TR, and an average DNA content per human cell of 6 pg.

**Western blotting** ECRF24 cells were homogenized in 0.25 M sucrose containing complete-protease inhibitor (Roche Molecular Biochemicals). Of this whole cell extract, 30 µg were loaded onto a 10% SDS-PAGE. After blotting (BA45; Schleicher and Schuell, Dassel, Germany) the blot was blocked in 5% non-fat dried milk in PBS for 1 h at room temperature. TR-isoform-specific rabbit polyclonal antisera in the same buffer were incubated for a further hour. Bands were visualized using goat anti-rabbit conjugated to horseradish peroxidase and LumiLightPlus substrate (Roche Molecular Biochemicals). Average exposure time on the LumiImager (Roche Molecular Biochemicals) was 5 min.

**Immunocytochemistry** Cells were grown on microscopic slides coated with gelatin. Before and after each of the following procedural steps, cells were washed twice with PBS. Fixation was performed with 4% paraformaldehyde for 15 min. After blocking for 30 min with 10% normal goat serum in PBS (pH 7.4) with 0.5% Triton X-100, the TR-isoform-specific rabbit polyclonal antisera in 10% normal goat serum in PBS with 0.5% Triton X-100 were added. Incubation was at room temperature for 60 min. Incubation with the second antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase, was at room temperature for 60 min. The pH was raised by incubation in buffer containing 0.1 M Tris, 0.1 M NaCl and 50 mM MgCl$_2$ (pH 9.2) for 10 min. Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals)+levamisole (Sigma), to inhibit endogenous alkaline phosphatase, were added for 20 min. Slides were covered with Kaiser glycerine medium and dried overnight. Detection of positively stained cells was with a light microscope. As a control, slides incubated with normal rabbit serum were used.

**ET-1 and vWF secretion by ECRF24 cells**

Cells were cultured in six-well plates until they were confluent. Then regular medium was replaced by serum-free medium for 24 h. Subsequently ET-1 production was measured in cell supernatant in relation to the amount of cells (0.25–, 0.50– and 1.0 $\times$ 10$^6$ cells), in relation to duration of culture (4, 8, 12 and 24 h) and in relation to the amount of T3 added (0 M, 10$^{-10}$–10$^{-6}$ M). Prior to ET-1 measurement, cell supernatant was extracted with C2 solid phase minicolumns (Amprep; Amersham International plc, Little Chalfont, Bucks, UK ) according to the manual of the manufacturer. ET-1 was measured
with a quantitative colorimetric two site immunoenzymometric ‘sandwich’ ELISA (Biotrak Endothelin-1 ELISA system, Amersham International) with a detection limit of 1 fmol/well. vWF was measured using an in-house sandwich-ELISA using rabbit anti-human vWF (A082; Dako, Copenhagen, Denmark) and rabbit anti-human vWF peroxidase conjugate (P226; Dako) with a pool of 100 normal human samples as a standard.

**Results**

**TR mRNAs**

We used real-time PCR to quantify the mRNAs encoding for the different isoforms of the TR in immortalized human endothelial cells. We found that there were about 1500 ag/20 µl TRα1, 160 ag/20 µl TRα2 and 8 ag/20 µl TRβ1. Calculating the approximate number of mRNA molecules per cell from these figures gives an estimate of at least 50 molecules per cell for TRα1, five for TRα2 and two for TRβ1 (Fig. 1).

**TR proteins**

Figure 2 shows the Scatchard plot of nuclear T₃ binding. The non-specific binding of [¹²⁵I]T₃ was 0·71% and the specific binding was 4·57% (580 µg DNA/tube). Specific T₃ binding increased linearly with the amount of nuclei (DNA) added. The MBC for T₃ of the endothelial cell nuclei was 55·2 fmol T₃/mg DNA, the association constant Kᵢ of T₃ was 8 × 10⁹ l/mol (Kᵢ 125 pmol/l). The number of TR protein molecules was calculated as approximately 200/cell.

Immunocytochemistry revealed perinuclear staining with anti-TRβ1, anti-TRα2 and anti-TRα1 (Fig. 3). No staining was observed with the respective pre-immune sera (data not shown).

The Western blot provided further evidence for the presence of the different TR isoforms. Bands corresponding to the expected sizes of the TRα1, TRα2 and TRβ1 isoforms, 47, 52 and 55 kDa respectively, were observed (Fig. 4).

**ET-1 and vWF production**

ET-1 production increased in proportion to the amount of cells (0·25–1·0 × 10⁶/well) from 6·4 to 20·4 fmol/well.
when cultured for 4 h. In time-course experiments ET-1 production increased from \( \approx 20 \text{ fmol/well} \) at 4 h to \( \approx 70 \text{ fmol/well} \) at 24 h. The addition of increasing amounts of T3 had no influence on ET-1 production at any time-point compared with cultures in the absence of T3 (Fig. 5a). vWF production by ECRF24 cells increased with time but no effect of T3 was observed (Fig. 5b). To check that the lack of effect of T3 was not due to decreased availability of the hormone, we measured free T3 in our culture medium. Under basal conditions this was about 4 pmol/l and when \( 10^{-6} \text{ M} \) T3 was added to the culture medium >70 pmol/l free T3 were measured.

**Discussion**

Using real-time PCR we were able to quantify for the first time the mRNA for TR\( \alpha_1 \) (50 copies/cell), TR\( \alpha_2 \) (five copies/cell) and TR\( \beta_1 \) (two copies/cell) isoforms expressed by the immortalized HUVEC line ECRF24. We found more TR\( \alpha_1 \) and TR\( \alpha_2 \) than TR\( \beta_1 \) mRNA, which is in accordance with previous results on the TR isoform mRNAs in bovine aortic endothelial cells (Hu et al. 1994). The amount of mRNA for the TR\( \alpha_1 \) (acting in a positive fashion on gene expression) appeared to be 10-fold higher than for the TR\( \alpha_2 \) (acting in a negative fashion on gene expression), which suggests that these endothelial cells are potentially T3 responsive when the corresponding proteins are translated.

At the protein level, TR\( \alpha_1 \), TR\( \alpha_2 \) and TR\( \beta_1 \) were all expressed, as evident from both immunocytochemistry and Western blots. The discrepancy between the low amount of mRNA for the TR\( \beta_1 \) and the apparent presence of TR\( \beta_1 \) protein is similar to that found for TR\( \beta_2 \) in rat liver (Ercan-Fang et al. 1996). One possible explanation could be a short half-life of the TR\( \beta_1 \) mRNA as was indeed suggested for the TR\( \beta_2 \) mRNA/protein discrepancy (Ercan-Fang et al. 1996).

The perinuclear staining pattern of the TR\( \beta_1 \) is in accordance with the site of action of the TRs, binding to their thyroid hormone response elements (TREs) on the DNA. Perinuclear and cytoplasmic staining of TRs has been reported in other studies on TR localization in both endothelial cells (Sellitti et al. 1985, Baumgartner-Parzer et al. 1997) and other cell types like hepatocytes (Chamba et al. 1996), extra-ocular muscle fibres (Schmidt et al. 1992) and anterior pituitary cells (Yen et al. 1992).

No increase in ET-1 or vWF production by ECRF24 cells after short-term exposure (maximum 24 h) to T3 was observed by us. Baumgartner-Parzer et al. (1997) reported that HUVECs were T3 responsive with regard to both ET-1 and vWF production. However, this was seen only after prolonged culturing (13 days), not after short-term
incubation (24 h). Other studies did demonstrate effects on ET-1 after short-term incubations with T₃. In rat aortic endothelial cells a slight increase (+17%) in ET-1 secretion after a 3–12 h incubation with T₃ at 10⁻⁶ M and an increase of adrenomedullin mRNA after 3 h incubation with 10⁻⁶ M T₃ have been found (Imai et al. 1995, Isumi et al. 1998). Furthermore, in bovine pulmonary artery cells a 2-fold increase in angiotensin-converting enzyme activity after 48 h incubation with 10⁻⁶ M T₃ has been reported (Krulwitz et al. 1984). One reason why we did not observe an effect on ET-1 in the ECRF24 cells could be the venous origin of our cells, in contrast to the other short-term studies, which used endothelial cells of arterial origin.

Assuming that a T₃-responsive gene contains one TRE and taking into account an average Kₛ of about 10⁻⁹ mol/l for a nuclear factor binding to its specific binding site (Bakker & Parker 1991), one can estimate that at least 1000 TR molecules per cell are needed to mediate T₃ responsiveness. Our Scatchard analysis showed that only ≈200 TR molecules were available per endothelial cell, which is far below this amount. The Kₛ of 125 pmol/l was in accordance with the magnitude of the Kₛ measured in bovine aortic endothelial cells (Hu et al. 1994). Comparison of the figure of 200 sites per ECRF24 cell with that in rat liver tissue, which has about 4000 TR sites/cell (Bakker & Parker 1991), also suggests that the number of TR sites per endothelial cell is probably not enough to mediate T₃ effects. Similar numbers of TRs have been found in human peripheral blood lymphocytes and placenta (Banovac et al. 1986, Matzen et al. 1989) but postreceptor effects have not been reported for these cells either.

In conclusion, the immortalized HUVEC line ECRF24 expresses TRα1 and TRα2 isoforms at both the mRNA and protein level, whereas for the TRβ1 there appears to be a discrepancy between its mRNA and protein levels. Although ≈200 TR sites per cell were found, we conclude that this amount is probably insufficient to mediate genomic T₃-induced effects, as was evident from the absence of an increase of ET-1 or vWF secretion upon incubation with T₃.

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The ECRF24 cell line was a kind gift from Ruud Fontijn (Department of Biochemistry, Academic Medical Centre, Amsterdam).

References


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