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 response to triiodothyronine (T_3) administration. A real-time PCR technique was used to quantify the presence of mRNAs encoding for the different isoforms of the TR. The binding of T_3 to nuclear TRs was studied in isolated endothelial cell nuclei by Scatchard analysis. Expression of TR at the protein level was investigated by immunocytochemistry and Western blotting using TR-isoform-specific polyclonal rabbit antisera. ET-1 and vWF were measured in cell supernatants with a two-site immunoenzymatic assay. Scatchard analysis yielded a maximum binding capacity of 55 fmol T_3 /mg DNA (± 200 sites/

cell) with a K_d of 125 pmol/l. Messenger RNAs encoding for the TR α 1 and the TR α 2 and the TR β 1 were observed. The approximate number of mRNA molecules per cell was at least 50 molecules per cell for TR α 1, five for TR α 2 and two for TR β 1. Immunocytochemistry revealed (peri)nuclear staining for TR β 1, TR α 1 and TR α 2. ET-1 and vWF secretion did not increase upon addition of T_3 (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 T_3 . Therefore we conclude that the genomic effects of T_3 are unlikely to occur in these immortalized HUVECs.

Journal of Endocrinology (2001) **168**, 427–433

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 (T_3) 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 α 1, α 2, β 1 and β 2, which are present in different tissues in different relative concentrations. TR α 2 is a splicing variant of the α gene that is not able to bind T_3 but may act in a dominant-

negative fashion on T_3 -dependent gene expression. TR α 1 and TR β 1 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 TR α 1, TR α 2 and TR β 1 were found using Northern blot and S1 nuclease protection. At the protein level, specific binding with $^{125}\text{I}[\text{T}_3]$ 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 TR α 1, TR α 2 and TR β 1 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 T_3 . In

cultured primary human umbilical vein endothelial cells (HUVECs) (peri)nuclear and cytoplasmic staining of TR β 1 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 T₃ (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 TR α 1 and TR α 2) and found TR α and TR β 1 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 immunocytochemistry to study TR isoform protein expression and looked for T₃-induced ET-1 and vWF expression (postreceptor effect).

Materials and Methods

Materials

RPMI 1640 (with Hepes and glutamine), Medium 199 (M199) (with Na₂HCO₃ and glutamine), human serum, L-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'-Triiodothyronine (T₃) was obtained from Henning GmbH, Berlin, Germany, [¹²⁵I]T₃ (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 (α 1, α 2 and β 1) 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 Vuyst, 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% CO₂. Culture medium consisted of equal volumes of RPMI 1640 (with Hepes and glutamine) and M199 (with Na₂HCO₃ 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 T₃ were measured in the culture medium using a Delfia free T₃ 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 $r=1.00$. 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 T_3 . To determine the association constant (K_a) and maximum binding capacity (MBC) of the binding of T_3 to the nuclear receptors, Scatchard analysis was performed. To this end increasing amounts of non-radioactive T_3 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 T_3 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 LumiLight^{Plus} 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

Figure 1 TR isoform mRNA levels in immortalized ECRF24 HUVECs. The plots show the fluorescent signal (F1), as measured using the LightCycler, of the standard curves (thin black lines) which were used to calculate the amount of RNA per cell in the sample (thick black line). The numbers at the standard curves indicate the mRNA amount per cell equivalent.

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 1.0×10^6 cells), in relation to duration of culture (4, 8, 12 and 24 h) and in relation to the amount of T_3 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

Figure 2 Scatchard plot of T_3 binding to nuclei of immortalized ECRF24 HUVECs. Equation of regression line: $y = 0.498 - 0.009x$, $r = -0.978$, $P = 0.02$.

with a quantitative colorimetric two site immunoassay-metric '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 μ l TR α 1, 160 ag/20 μ l TR α 2 and 8 ag/20 μ l TR β 1. Calculating the approximate number of mRNA molecules per cell from these figures gives an estimate of at least 50 molecules per cell for TR α 1, five for TR α 2 and two for TR β 1 (Fig. 1).

TR proteins

Figure 2 shows the Scatchard plot of nuclear T_3 binding. The non-specific binding of [125 I] T_3 was 0.71% and the specific binding was 4.57% (580 μ g DNA/tube). Specific T_3 binding increased linearly with the amount of nuclei (DNA) added. The MBC for T_3 of the endothelial cell nuclei was 55.2 fmol T_3 /mg DNA, the association constant K_a of T_3 was 8×10^9 l/mol (K_d 125 pmol/l). The number of TR protein molecules was calculated as approximately 200/cell.

Immunocytochemistry revealed perinuclear staining with anti-TR β 1, anti-TR α 2 and anti-TR α 1 (Fig. 3). No

Figure 3 The expression of TR proteins in immortalized ECRF24 HUVECs. Cells were grown on slides and incubated with (a) TR β 1-, (b) TR α 2- and (c) TR α 1-specific antipeptide rabbit polyclonal antibodies. For all isoforms perinuclear staining is observed.

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 TR α 1, TR α 2 and TR β 1 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 – 1.0×10^6 /well) from 6.4 to 20.4 fmol/well

Figure 4 Western blot of whole cell extracts of ECRF24 cells. Parts of the blot were incubated with TR α 1-, TR α 2- and TR β 1-specific antipeptide rabbit polyclonal antibodies. Bands were detected using LumiLight^{plus} horseradish peroxidase substrate. Their positions correspond to the expected length of the TR α 1, TR α 2 and TR β 1 isoforms, at 47, 52 and 55 kDa respectively.

when cultured for 4 h. In time-course experiments ET-1 production increased from ≈ 20 fmol/well at 4 h to ≈ 70 fmol/well at 24 h. The addition of increasing amounts of T_3 had no influence on ET-1 production at any time-point compared with cultures in the absence of T_3 (Fig. 5a). vWF production by ECRF24 cells increased with time but no effect of T_3 was observed (Fig. 5b). To check that the lack of effect of T_3 was not due to decreased availability of the hormone, we measured free T_3 in our culture medium. Under basal conditions this was about 4 pmol/l and when 10^{-6} M T_3 was added to the culture medium >70 pmol/l free T_3 were measured.

Discussion

Using real-time PCR we were able to quantify for the first time the mRNA for TR α 1 (50 copies/cell), TR α 2 (five copies/cell) and TR β 1 (two copies/cell) isoforms expressed by the immortalized HUVEC line ECRF24. We found more TR α 1 and TR α 2 than TR β 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 α 1 (acting in a positive fashion on gene expression) appeared to be 10-fold higher than for the TR α 2 (acting in a negative fashion on gene expression), which suggests that these endothelial cells are potentially T_3 responsive when the corresponding proteins are translated.

At the protein level, TR α 1, TR α 2 and TR β 1 were all expressed, as evident from both immunocytochemistry and Western blots. The discrepancy between the low amount of mRNA for the TR β 1 and the apparent presence of TR β 1 protein is similar to that found for TR β 2 in rat liver (Ercan-Fang *et al.* 1996). One possible explanation could be a short half-life of the TR β 1 mRNA as was indeed suggested for the TR β 2 mRNA/protein discrepancy (Ercan-Fang *et al.* 1996).

The perinuclear staining pattern of the TR β 1 is in accordance with the site of action of the TRs, binding to

Figure 5 Postreceptor effects in immortalized ECRF24 HUVECs. (a) ET-1 production in relation to the duration of incubation (4, 8, 12 and 24 h) and in relation to the amount of T_3 added to the culture medium. (b) vWF production in relation to time and T_3 (at 10^{-6} M, triangles). Control incubations are shown by the black dots.

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 T_3 was observed by us. Baumgartner-Parzer *et al.* (1997) reported that HUVECs were T_3 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 T_3 . In rat aortic endothelial cells a slight increase (+17%) in ET-1 secretion after a 3–12 h incubation with T_3 at 10^{-6} M and an increase of adrenomedullin mRNA after 3 h incubation with 10^{-6} M T_3 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 T_3 has been reported (Krulowitz *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 T_3 -responsive gene contains one TRE and taking into account an average K_d 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 T_3 responsiveness. Our Scatchard analysis showed that only ≈ 200 TR molecules were available per endothelial cell, which is far below this amount. The K_d of 125 pmol/l was in accordance with the magnitude of the K_d 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 (Ercan-Fang *et al.* 1996), also suggests that the number of TR sites per endothelial cell is probably not enough to mediate T_3 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 TR α 1 and TR α 2 isoforms at both the mRNA and protein level, whereas for the TR β 1 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 T_3 -induced effects, as was evident from the absence of an increase of ET-1 or vWF secretion upon incubation with T_3 .

Acknowledgement

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

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Received 23 October 2000

Accepted 31 October 2000