BIOMEDICAL DATA JOURNAL
datajournals.eu

Sabatino L et al. *Biomed Data J.* 2015; 1(2): 19-25
http://dx.doi.org/10.11610/bmdj.01203

ISSN 2367-5322, e-ISSN 2367-6477

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Data Paper

Thyroid Hormone Deiodinases and Receptors Are Expressed in Human Endothelial Cells

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ARTICLE INFO:

RECEIVED: 18 Oct 2015
REVISED: 13 Nov 2015
ACCEPTED: 28 Nov 2015
ONLINE: 15 Dec 2015

KEYWORDS:
thyroid hormones
deiodinases
endothelial cells

ABSTRACT

The endothelial system acts as a large endocrine organ in the human body, however, little is still known about the regulative role of THs on endothelial cells. The present study aims to investigate the expression of the TH deiodinases (D1, D2, D3) and TH receptors (TRα1, TRα2 and TRβ1) in an endothelial microvascular cultured cell model (HMEC-1), after stimulation with triiodothyronine (T3, 10-100nM), thyroxine (T4, 10-100nM), and reverse T3 (rT3, 1-10nM). The main result of the study is that the TH deiodinases and receptors are expressed in endothelial HMEC-1 cells. These findings might be of significant clinical relevance, given the important regulatory role of the endothelium as first barrier to the bloodstream.

Overview

Endothelium can be considered a major endocrine organ in the human body. The effects of THs on endothelial cells are still far from being totally elucidated. Endothelium has a relevant role in atherosclerosis and related cardiovascular diseases and altered thyroid function is often associated with increased risk of cardiovascular events.[1] TH deiodination is a crucial step in the modulation of TH signalling in the cells and the deiodinases are the regulatory enzymes involved in the process.[2] After entering the cells through specific transporters, THs are metabolized by deiodinases and diffuse into the cell nucleus, interact with specific receptors, thus regulating the expression of TH-dependent genes.[2-4]

Conversion of T4 to the biologically active form T3 occurs by outer ring deiodination in 5' position, and is mediated by two specific deiodinases (D1 and D2). D3 enzyme is responsible for deactivation of THs through deiodination on the 5 position of the inner ring of the molecule and the product of 5 deiodination of T4 is rT32. Moreover, D1 is the sole enzyme that can function as either an outer or an inner ring deiodinase, determining in turn activation or deactivation of THs.[5,6] The initial studies located the D1 expression mainly in the liver and the kidneys,[7] the D2 in the anterior pituitary and the central nervous system and D3 in placenta and skin.[8] Successive investigations demonstrated a more widespread distribution of the deiodinases in various other tissues than initially believed, suggesting the hypothesis that the counterbalancing of the different deiodinases in the TH-target cells is the key factor for a tissue-specific TH signaling.[9,10] Furthermore, different cell lines, such as cultured rat astrocytes and thyroid-

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derived FRTL-5,[11] mouse skeleton and mouse osteoblastic cells,[12] have been set up in order to better clarify the cellular processing and signalling cascades affecting 5' deiodinase expression and activity under different conditions and stimuli. Huang et al reported evidence on D3 expression in endothelial cells of fetoplacental unit[13] and in the endothelium of infantile hemangiomas.[14,15] However, to the best of our knowledge, no investigations have been performed on the expression of 5' deiodinases in the endothelial system.

The aim of the present study was to investigate the expression of D1, D2, D3 and TRα1 and TRα2 in an endothelial dermal microvascular cultured cell model (HMEC-1) after stimulation with different concentrations of THs.

In this report, we present a dataset obtained from HMEC-1 cell line after stimulation with different concentrations of THs. Parts of these data belongs to a previous published work.[16]

Methods

Cell Culture

Human endothelial dermal microvascular cell line HMEC-1, firstly set up by Ades et al,[17] was chosen as model for the study (Life Technology, Monza, Italy). Cells were cultured in M-199 medium (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% glutamine, 1% antibiotics, 1% epidermal growth factor (EGF; Sigma Chemical Co., St. Louis, MO) and 0.5% hydrocortisone. Cells were cultured at 37°C in 5% CO2, and the medium was replaced every two days.

TH treatment

HMEC-1 cells were incubated with TH free-FBS for three hours before treatment with the hormone. All the hormones were purchased from Sigma, Milan, Italy. For each hormone treatment, untreated cells and two different concentrations were used on the basis of what was already reported in the literature on other cell types.[18-20] In T3 experiments, cells were treated with 10 nM or 100 nM of hormone; in rT3 experiments, 1 nM or 10 nM concentrations were used and in T4 experiments, cells were treated with 10 nM or 100 nM of hormone. Cells were cultured at 37°C in 5 % CO2 for 24 hours. In each experiment, an equal volume of cell suspension was kept in the absence of hormone and was used as untreated control. Analysis for each hormonal treatment was performed on 6 replicates.

RNA extraction and reverse transcription

After 24 hours of incubation with the TH, cells were collected with 0.7 ml of Qiazol (Qiagen, Milan, Italy) and total RNA extracted with miRNeasy Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. A quantity of 1 μg of total RNA obtained from each sample was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy), according to the manufacturer's instructions.

Quantitative Real-Time PCR analysis of gene expression

Real-Time PCR reactions were performed in a 384-well CFX384 RT-PCR System (Bio-Rad, Milan, Italy). Reaction mixture included 4 μl of template cDNA (1:5 diluted), 0.5 μM of each primer and 2X iTaq Universal Sybr Green Supermix (Bio-Rad, Milan, Italy). Amplification protocol started with 95°C for 30 seconds followed by 39 cycles at 95°C for 5 seconds and 60°C for 15 seconds. To assess product specificity, amplicons were checked by melting curve analysis. Melting curves were generated from 65°C to 95°C with increments of 0.5°C/cycle. Each assay was performed in triplicates, with negative control. The combination of GeNorm and qBase software technology following recent guidelines was used to assess the expression stability of each candidate reference gene and to determine the ideal number of genes required for normalization in order to calculate individual normalization factors for each sample. The average Ct values obtained from each triplicate was converted to a relative quantity and analysed using the CFX384 Manager algorithm. Gene stability is expressed by the M value, which is calculated as the average variation between one of the genes and all the others analysed. Genes are ranked for their M value and at each step of the analysis the least stable gene (highest M value) is excluded and M is recalculated. The most stable genes are identified and internal control genes with M value < 0.5 were chosen for normalization. The analysis indicated the four most stably expressed housekeeping genes: HPRT-1: Hypoxanthine phosphoribosyltransferase I; TPT1: Translationally-controlled tumour protein 1; RPL13A: Ribosomal Protein L13a; and EEF1A: Eukaryotic translation elongation factor 1A. Primers' details of reference and target genes are shown in Table 1. The genes for the three deiodinases are indicated as DIO1, DIO2 and DIO3. A standard curve for each target and housekeeping gene was evaluated to assess amplification efficiency and linearity.

Table 1. Reference and target genes: primer details.

Gene	Primer sequence (5'–3')	Length bp	Efficiency %	GenBank n
TPT1	F: AAATGTTAACAAATGTGGCAATTAT R: AACAATGCCTCCACTCCAAA	164	108	NM_003295.2
RPL13A	F: CGCCCTACGACAAGAAAAAG R: CCGTAGCCTCATGAGCTGTT	206	107	NM_012423.2
EEFIA1	F: CTTTGGGTCGCTTTGCTGTT R: CCGTTCTTCCACCACTGATT	183	98	NM_001402.5
HPRT-1	F: TGCTGACCTGCTGGATTACAT R: TTGCGACCTTGACCATCTTT	259	113	NM_000194.2
DIO1	F: AGCCACGACAACTGGATACC R: ACTCCCAAATGTTGCACCTC	159	105	NM_BC107170.2
DIO2	F: CCACATGCCACCTTCTTGACTT R: CCCGTAAGCTATGTTGGCGTTA	343	91	NM_BC063118.1
DIO3	F: GCCTACTTCGAGCGTCTCTATG R: CATAGCGTTCCAACCAAGTGCG	112	105	NM_001362.3
TRα1	F: GGTGCTGCATGGAGATCATG R: GGAATGTTGTGTTTGCGGTG	325	102	NM_M24748.1
TRα2	F: GGTGCTGCATGGAGATCATG R: TCGATCTTGTCCACACACAG	259	106	NM_J03239.
TRβ1	F: CGGAGGAGAAGAAATGTAAAGG R: GCTTCGGTGCAGTTTTGATG	421	103	AK_096628

Western Blotting

Cell lysate proteins (10-20 μg) were resolved by Bolt 4-12 % gradient mini gels using the Bolt mini gel electrophoresis system (Life Technologies, Monza, Italy). Gels were blotted onto 0.2 mm PVC-membrane by iBlot Dry Blotting System (Life Technologies, Monza, Italy). Membranes were incubated with specific polyclonal Antibodies (Santa Cruz, Milan, Italy), and the appropriate secondary IgG-HRP linked conjugate antibody was applied. Proteins were visualized with a chemiluminescence assay (Euroclone, Milan, Italy) and the optical density (OD) of specific bands analysed with ImageJ Software. Enzymes are indicated as D1 and D2 and the results are expressed as D1 or D2 OD normalized to the reference protein gapdh OD. For each enzyme, final data were obtained from an average of three assays.

Data Records

Table 1 below presents the data structure in file 01203_Thyroid_hormone_deiodinases.xls.

The data in the accompanying spreadsheet is organised in the following columns:

A: Samples of the 9 groups of treatment with thyroid hormones, T3, rT3 and T4. UN (T3/rT3/T4) = untreated cells relative to T3, rT3 and T4 treatments; 10 nM or 100 nM of T3; 1 nM or 10 nM of rT3; 10 nM or 100 nM of T4.

B: DIO1 gene expression in the samples from the 9 groups.

C: DIO2 gene expression in the samples from the 9 groups.

D: DIO3 gene expression in the samples from the 9 groups.

E: TRα1 gene expression in the samples from the 9 groups.

F: TRα2 gene expression in the samples from the 9 groups.

G: TRβ1 gene expression in the samples from the 9 groups.

H: D1 protein levels gene expression in the samples from the 9 groups.

I: D2 protein levels gene expression in the samples from the 9 groups.

Validation

The dataset published in this paper was obtained after TH treatment of the endothelial microvascular cell line HMEC-1. The expression of TH deiodinases and receptors strongly suggest a direct physiological involvement of the endothelium as specific target for THs. This is extremely relevant from a functional point of view, given the role of the endothelial system as first barrier for circulating THs to crossing and leaving the bloodstream. Moreover, HMEC-1 is considered a valid and reliable model in the investigations on the human endothelial system, as already reported elsewhere.[17,21]

Our findings on gene expression indicated that all deiodinases are under TH regulation in HMEC-1 cells. It is commonly believed that D1 activity is regulated by THs almost exclusively at transcriptional level.[22-24] D1 enzyme is the only deiodinase with both 5'- and 5-deiodination activity, inducing activation or deactivation of THs. Besides the T4 to T3 conversion/activation, D1 is responsible of deactivation of excessive rT3[25] and this might at least in part explain the mRNA increase of DIO1 in cells treated with higher levels of rT3. However, it is not clear why D1 protein levels did not increase accordingly; a possible explanation could be that D1 half-life is 12 hours and it is known that subsequent degradation is enhanced by substrates such as iopanoic acid and rT3.[26] Moreover, a prolonged exposure of cells to TH-free medium could be more appropriate in order to guarantee a clean up of the cells from pre-existing deiodinase proteins (in particular D1 and D3 since they have longer half-lives), which is of relevant importance in the study of protein content and activity.

D2 enzyme has only a 5'-deiodination activity and its expression shows adaptive changes in response to alterations in thyroid state, which serve to maintain tissue T3 levels in the face of varying plasma T4 and T3 levels. These findings have led to the general opinion that D2 is important for the generation of local T3.[27] In contrast to D1, the control of the D2 expression is more complex, occurring by transcriptional, post-transcriptional, and post-translational mechanisms.[28-31] Contrasting data on D2 regulation by THs were obtained from various in vitro and in vivo studies. In cultured rat adipocytes treated with T3, an increase of D2 mRNA was observed, but no change in activity was detected.[32] Interestingly, in the brain and the brown adipose tissue of T3-treated mice a decrease of D2 mRNA was detected whereas D2 activity was induced. This result was probably due to the decreased serum T4 levels.[33] Furthermore, other experiments demonstrated that the administration of a combination of T3 plus T4 abolished the increase of D2 activity determined by T3 in absence of T4.[33] These studies pointed out how the different THs can reciprocally interfere in the regulatory processes and this aspect is crucial for interpretation of data when working in a cell system which is exposed to only one hormone and its possible products. In our cell system, DIO1 and DIO2 did not change after T3 administration and they both were significantly reduced after T4 administration. To better understand these effects, it might be useful to investigate also the cellular response to the combination of different THs.

Previous studies on regulation at post-translational levels demonstrated that a major D2 property that confirms its homeostatic relevance is the short half-life (30-40 min) that can be further reduced after prolonged exposure to D2 preferred substrates, T4 and rT3.[33] Moreover, the cellular events that lead to this substrate-mediated inactivation involve an ubiquination pathway which promotes D2 degradation by proteasomes.[30,34]

Studies on D3 showed that it is positively regulated by THs and represents a powerful mechanism by which TH-inactivation participates in TH-homeostasis in the presence of excessive levels of hormones. THs regulate DIO3 expression at transcriptional level in several systems.[35,36] D3 is often involved in inflammatory processes where altered hormonal levels are commonly detected and D3 generally operates to control the bioavailability of T3.[37]

In our study both T3 and rT3 excess induced an over-expression of DIO3 in HMEC-1 cells. At higher levels of rT3 we observed also an increase of DIO1 and a reduction of DIO2 expression, which is in agreement with the necessity to inactivate the overloaded of cellular rT3.

TRβ1 expression significantly increased after treatment with T3. This result is not surprising if

compared to what was previously seen by Kinugawa et al in cardiomyocytes.[38] Unlike cardiomyocytes, where no increase in TRα1 and TRα2 after treatment with 100nM T3 was observed, we found that TRs were all T3-responsive at high T3 dosage. The possibility of a different effect needs to be evaluated also at post-translational level where the involvement of specific co-factors may determine a different final response by the cell.

Use and Potential Reuse of the Dataset

On the basis of data from literature, the role of endothelium and smooth muscle cells in the response to excessive THs is controversial and some authors pointed at smooth muscle cells rather than endothelium as main target for THs.[39-41] Previous studies have shown direct and acute effects of circulating T3 on the resistance vessels by stimulating endothelial function and vasoconstriction norepinephrine-mediated.[42] Furthermore, data obtained in rat aortic rings indicated that THs exert part of their vascular effects also through a nitric oxide activity on endothelium and that the response to increased acetylcholine levels did not involve smooth muscle cells.[21]

The aim of the present study was to investigate the presence and regulation of TH deiodinases and receptors in endothelial cells using the microvascular model HMEC-1. It is reasonable to presume that in vitro conditions do not permit to analyse the integrated response of peripheral microcirculation and the real relative role of endothelium in the regulation by THs. On the other hand, the in vitro approach allows to define unequivocally the hormonal effects at one-cell type level, since all interferences from other cellular systems are abolished.

Thus, open-access dataset referring to the deiodinase response after TH stimulation in the endothelium could be a valuable tool to better understand the adaptation of this cellular context to TH through the development of computational models. Furthermore, modelling the response in the endothelium of the three deiodinases at transcriptional and post-transcriptional levels could help to provide new insights on the different reciprocal role of the three enzymes after specific hormonal stimulations.

In perspective, interesting in-depth information on the endothelial role of deiodinases and TH effects in humans could be derived by investigating the expression, protein levels and enzymatic activities of the three enzymes in different endothelial context.

Conclusion

Our data indicate for the first time that the three TH deiodinases are present in endothelial cells and that they are targeted by THs. These findings might be of extreme clinical relevance in physiological and pathological conditions, given the important regulatory role of the endothelium as first barrier to the bloodstream.

Data Files

The data described in this paper is presented in an openly accessible spreadsheet “01203_Thyroid_hormone_deiodinases.xls” (and the accompanying file in the Open Document Spreadsheet format with extension .ods).

Acknowledgement

The preparation of this manuscript was supported by the EU-funded project OpenScienceLink (Grant agreement # 318652).

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