Atherosclerosis 218 (2011) 77–82

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Thyroid hormone enhances the ability of serum to accept cellular cholesterol via the ABCA1 transporter

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ARTICLE INFO

Article history:
Received 18 December 2010
Received in revised form 5 April 2011
Accepted 26 April 2011
Available online 6 May 2011

Keywords:
Thyroid hormone
Atherosclerosis
Cholesterol transport
ABCA1 transporter

ABSTRACT

Objective: The goal of this study was to examine the effects of thyroid hormone status on the ability of serum to accept cellular cholesterol.
Methods and results: Sera from hypophysectomized rats treated ±T_3 was used to evaluate the role of thyroid hormone on serum efflux capacity. 2D-DIGE analysis of serum proteins showed that T_3 treated rats had increased ApoA-I, ApoA-IV and fetuin A levels with decreased Apo E levels. Microarray and real-time RT-PCR analysis of rat liver revealed large increases in ApoA-I, ApoA-IV, ABCG5, and ABCG8 in response to T_3. J774 macrophages, BHK cells, and Fu5AH rat hepatoma cells were used to measure cholesterol efflux mediated by ABCA1, ABCG1 transporters or SR-BI. Sera from T_3-treated rats stimulated efflux via ABCA1 but not by ABCG1 or SR-BI. Gel filtration chromatography revealed that T_3 treatment caused a decrease in HDL particle size accompanied by higher levels of lipid-poor ApoA-I.
Conclusions: Thyroid hormone enhances the ability of serum to accept cellular cholesterol via the ABCA1 transporter. This effect is most likely attributable to increases in small HDL and lipid poor ApoA-I in response to T_3.

1. Introduction

It is well established that thyroid hormone acts to lower serum cholesterol levels [1,2]. The mechanism by which this occurs appears to involve the modulation of several key processes in cholesterol metabolism. These likely include: increased elimination of neutral sterols and bile acids from the body, decreased rates of intestinal absorption of cholesterol, and increased removal of LDL cholesterol from the circulation by the liver [3,4]. These findings have lead to the investigation of thyroid hormone mimetic compounds, thyromimetics, as a treatment for hypercholesterolemia [5,6]. Recent clinical trials have found that thyromimetics are capable of lowering plasma LDL cholesterol and increasing bile acid synthesis in the absence of negative cardiac side effects [7]. Furthermore, animal studies utilizing the liver-selective thyromimetic T-0681 have shown that treatment with T-0681 promotes reverse cholesterol transport as measured by fecal sterol excretion [8]. Additional knowledge on the role of thyroid hormone in foam cell formation and cholesterol efflux will be beneficial to the future clinical use of thyromimetics in the treatment of hypercholesterolemia and its associated diseases [9]. However, until now, there has been very little information regarding the effects of thyroid hormone on cholesterol efflux capacity – the quintessential anti-atherogenic property of HDL.

Low HDL levels are a strong independent risk factor for death from cardiovascular disease, however, HDL cholesterol levels may be an inadequate indicator of HDL function. Strategies that enhance the anti-atherogenic properties of HDL, particularly its ability to promote cholesterol efflux from the macrophage and thereby drive reverse cholesterol transport, are being actively pursued [10]. The contributions of the ABCA1 and ABCG1 transporters in macrophage cholesterol transport to HDL and ApoA-I are well documented [11,12]. In this study, we sought to examine the effects of thyroid hormone status on the ability of HDL to promote cholesterol efflux from the macrophage. Using two hypothyroid rat models, we investigated the effect of thyroid hormone on ABCA1, ABCG1, and SR-BI-mediated cholesterol efflux *ex vivo*. We report that serum from thyroid hormone treated animals promotes cholesterol efflux via the ABCA1 transporter but not ABCG1.

2. Methods

2.1. Experimental animals

Hypophysectomized, thyroidectomized and normal male Sprague-Dawley rats weighing 125–150 g were purchased from

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doi:10.1016/j.atherosclerosis.2011.04.028

Harlan (Indianapolis, IN). Hypophysectomized rats received Tekland iodine deficient chow and water ad libitum and were housed in a reverse-cycle light controlled room with a 12-h light period followed by a 12-h dark period. The animals were cared for according to the NIH guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" and specifically in accord with protocol 35 71 approved by the University of South Florida Institutional Animal Care and Use Committee. Thyroidectomized rats received 1% calcium gluconate as their drinking water in addition to the iodine deficient chow. The rats were maintained on the iodine deficient diet for at least 14 days prior to being used in experiments in order to achieve sufficient turnover of T_4. The normal rats were fed Tekland 22/5 rodent chow ad libitum. Hypophysectomized and thyroidectomized rats were given an initial injection of 1.0 mg/kg T_3 72 h prior to harvest of tissue and an additional injection of 0.25 mg/kg T_3 24 h prior to harvest of tissue. In both hypothyroid animal models, administration of T_3 alone restored cholesterol efflux via ABCA1 transporters, indicating that other pituitary regulated hormones are not required

2.2. Cholesterol, LDL/VLDL, HDL and T_3 assays

Blood was collected and centrifuged at 16,000 × *g* for 5 min. The supernatant (serum) was collected and used for these determinations. HDL levels were measured using the HDL and LDL/VLDL Cholesterol Quantification Kit from BioVision (Cat# K613-100; Mountain View, CA). T_3 levels were measured using the Free T_3 ELISA from Calbiotech (Cat# F3106T; Spring Valley, CA)

2.3. RNA isolation

A portion of liver (about 200 mg) was quickly excised and immediately homogenized in 4 ml of Tri-Reagent from Molecular Research Center (Cincinnati, OH) using a Polytron homogenizer at room temperature. The remainder of the isolation steps was carried out using volumes corresponding to 4× the manufacturer's recommendations

2.4. Microarray analysis

Isolated RNA was further purified using the RNeasy kit from Qiagen. RNA integrity was confirmed and microarray analysis was performed by the Moffitt Core Facility (Tampa, FL) using the Affymetrix GeneChip instrument system following the protocol established by Affymetrix, Inc. Ten microgram each of RNA from the livers of 3 Hx and 3 Hx + T_3 rats was used in the analysis. An Affymetrix GeneChip Rat Genome 230 Plus 2.0 arrays, which detects about 28,000 genes was used for the analysis. Multiple oligos are used for each gene with the data averaged. Scanned chip images were analyzed using GeneChip algorithms.

2.5. Real time PCR

To validate the microarray results, we assessed the expression of a subset of genes using real-time PCR. RNA was DNAse treated using the TURBO DNA-Free Kit (Ambion, Austin, TX). cDNA was prepared using the Reverse Transcription System (Promega) per the manufacturer's protocol. The primer sequences are listed in Supplementary materials. Hepatic mRNA was quantified under the following reaction conditions: 95 °C for 5 min, followed by 40 cycle of 95 °C for 15 s, 61 °C for 1 min and melt curve 55 °C + 0.5 °C each 10 s, ×80. All samples were run in duplicate on a Bio-Rad Chromo4 DNA Engine thermal cycler using SYBR green chemistry. Relative mRNA was calculated as a function of the internal control 18 s using ΔΔ Ct.

2.6. Cell culture and efflux studies

Efflux studies were performed as previously described [13] using either J774 macrophages, Fu5AH rat hepatoma cells, or BHK cells expressing ABCG1 (gift from Dr. Jack Oram, University of Washington School of Medicine). J774 cells were grown in 20% FBS-RPMI, Fu5AH cells in 10% CS-EMEM and BHK-GI cells in 10% FBS-DMEM in the presence of antibiotics. For efflux, cells were plated in 24-well plates (J774 and Fu5AH: 70,000 cells/well; BHK: 125,000 cells/well) and radiolabeled using [1,2-^{3}H] cholesterol (PerkinElmer. J774: 2 mci/ml in 5% FBS-RPMI; Fu5AH: 2 mCi/ml in 5% CS-EMEM and BHK: 1 mCi/ml in 2.5% FBS-DMEM) in the presence of an ACAT inhibitor (2 μg/ml, CP 113,818, a gift from Pfizer), also present during efflux, to prevent accumulation of cholesteryl esters. J774 cells were treated with 0.3 mM c-AMP (cpt-AMP, Sigma) in 0.2% BSA for 16–18 h to upregulate ABCA1. BHK cells were treated with 10 nM mifepristone for 16–18 h to upregulate ABCG1. Fu5AH were pretreated for 2 h with MEM-2% BSA + 1 μM BLT to inhibit SR-BI. All efflux medium was prepared using serum from ≥4 Tx or Hx rats treated ±T_3 diluted to 2% in MEM-HEPES and sera were assayed in triplicate. Efflux was measured as the fraction of total radiolabeled cell cholesterol released to the medium in 4 h and is corrected for the small amount released to MEM. In all experiments we also measured efflux to a pool of human serum diluted to 2%. ABCA1 efflux is considered to be the difference in efflux between control and c-AMP treated J774 cells and ABCA1 expression was verified by increased efflux to 20 μg/ml ApoA-I from c-AMP treated cells. SR-BI efflux is the BLT-1 sensitive efflux calculated from cells treated ±BLT and the inhibitor effect was monitored as loss of efflux to 25 μg/ml HDL_3. ABCG1 efflux is considered to be the difference in efflux between control and mifepistrone treated BHK cells and ABCG1 expression was verified by increased efflux to 25 μg/ml HDL_3 from mifepristone-treated cells.

2.7. 2D-DIGE

Two-dimensional differential in-gel electrophoresis was performed by Applied Biomics (Hayward, CA). Briefly, equal volumes of serum from 3 Hx rats or 3 Hx + T_3 rats were combined to form a pool for each treatment. A pool of serum from 2 normal rats was included as a control. Each pool was fluorescently labeled with different CyDye (Hx + T_3 – red, Hx – green, normal-blue) for downstream visualization. The samples were run on first dimension isoelectric focusing and second dimension SDS-PAGE. Fluorescent images were captured using a Typhoon image scanner and analyzed using ImageQuant software. Differentially expressed proteins were quantified using DeCyder software analysis, cut out, and subjected to in-gel trypsin digestion followed by protein identification by MALDI-TOF mass spectrometry

2.8. Fast performance liquid chromatography (FPLC) separation of lipoproteins

Pooled rat sera (150 μl) was diluted in running buffer (150 mM NaCl, 1 mM EDTA pH 8.0) and run over a two superpose 6 10/300 GL columns arranged in tandem (Pharmacia Biotech). A total of forty-six fractions (500 μl each) were collected and assayed for total cholesterol using the Cholesterol E kit from WAKO. The following antibodies were used for western blotting of FPLC fractions, serum and livers: rabbit anti-mouse Apolipoprotein A-I Biodesign K23500R, rabbit anti-ApoE Biodesign K23100R, a polyclonal antibody to the LDL receptor [4], and mouse monoclonal anti-ABCA1 (Abcam ab18180)