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Hypothyroidism Modifies Lipid Composition of Polymorphonuclear Leukocytes

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Key Words

Hypothyroidism • Triacylglycerides • Cholesterol • HMGCoAR

Abstract

Thyroid hormones are important regulators of lipid metabolism. Polymorphonuclear leukocytes (PMN) are essential components of innate immune response. Our goal was to determine whether hypothyroidism affects lipid metabolism in PMN cells. Wistar rats were made hypothyroid by administrating 0.1 g/L 6-propyl-2-thiouracil (PTU) in drinking water during 30 days. Triacylglycerides (TG), cholesterol and phospholipids were determined in PMN and serum by conventional methods. The mRNA expression of LDL receptor (LDL-R), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR), sterol regulatory element binding protein 2 (SREBP-2), and diacylglycerol acyltransferase 2 (DGAT-2) were quantified by Real-Time PCR. Cellular neutral lipids were identified by Nile red staining. We found hypothyroidism decreases serum TG whereas it increases them in PMN. This result agrees with those observed in Nile red preparations, however DAGT-2 expression was not modified. Cholesterol synthesizing enzyme HMGCoAR mRNA and protein was reduced in PMN of hypothyroid rats. As expected, cholesterol content decreased in the cells although it increased in serum. Hypothyroidism also reduced relative contents of palmitic, stearic, and arachidonic acids, whereas increased the myristic, linoleic acids, and the unsaturation index in PMN. Thus, hypothyroidism modifies PMN lipid composition. These findings would emphasize the importance of new research to elucidate lipid-induced alterations in specific function(s) of PMN.

Introduction

Hypothyroidism is one of the most-frequently-observed pathological conditions in the general population, its incidence being higher in women than in men [1].

It is well known that thyroid dysfunction leads to changes in lipoprotein metabolism [2]. Low-density lipo-

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protein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels increase in the plasma of hypothyroid patients. On the contrary, LDL-C and HDL-C decrease in hyperthyroidism. Furthermore, clearance of chylomicron remnants is reduced in hypothyroidism [3].

Induction of hypothyroidism in rats increases concentrations of total cholesterol (TC) in serum and cholesterol in low-density lipoproteins (LDL) [4]. Cholesterol content changes in high density lipoproteins (HDL) by the hypothyroidism are a controversial question that remains to be addressed. Some authors have reported that HDL-C levels increased [5], other authors did not find changes in HDL-C levels [6], while others found diminished HDL-C concentrations [7]. The TG concentration decreased in the serum of hypothyroid rats [2, 7, 8].

In addition, it has been demonstrated that hypothyroid condition decreased the mRNA levels of apoA-I and apoA-IV in rat liver suggesting that thyroid hormones may play an important role in regulating the mRNA levels of specific apolipoproteins [9].

So far, it is known that the immune system protects host from environmental infectious agents, such as pathogenic bacteria, viruses, fungi, parasites and other noxious insults. Innate immunity is the first line of defence against infectious agents and it consists of physical barriers (e.g. skin), soluble factors (e.g. complement) and phagocytic cells like neutrophils, monocytes and macrophages [10]. Neutrophils are released into the bloodstream -where they circulate- and die by apoptosis after a half-life of approx. 7 hours. They can also respond to signals of localized infection, migrate through the vascular endothelial walls into tight tissue spaces seeking out the infectious agents for phagocytosis and destruction [11, 12]. The lipid composition is very important in phagocytes for their protective functions. It has been demonstrated that changes in the membrane lipid composition of macrophages alter the clearance of apoptotic cells by regulating their phagocytic rate through phosphatidylinositol 3-kinase signaling [13].

There is a connection between immune function and lipid metabolism. It is well known that cholesterol plays a key role in membrane structure, and that it is a major player in the lipid rafts which have a vital role in cell signalling and protein sorting on the membrane surface [14]. Moreover, the expression of functional LDL-R was assessed by flow cytometry in PMN, monocytes and lymphocytes by Lara and coworkers [15]. These authors found that LDL-R expression and avidity are higher in PMN compared with mononuclear cells. Bonneau et al. [16] showed that low-density lipoproteins increase the production of superoxide anion and inhibit the chemotactic response of PMN to C5a and formyl-methionyl-leucyl-phenylalanine. These observations would indicate that PMN's functions could be altered by the environmental conditions in which they are embedded, including the presence of lipoproteins. In the same way, changes in membrane cholesterol and/or phospholipids fatty acid composition might be expected to influence immune cell function in a variety of ways. For instance, *in vitro* studies have clearly demonstrated that alterations in phagocyte membrane fatty acid composition are associated with altered phagocytic capacity, T-cell signaling and antigen presentation capability. These effects appear to be mediated at the membrane level, suggesting fatty acids play important roles in membrane arrange, lipid raft structure and function, and membrane trafficking [10].

To our knowledge, this is the first work that studies the influence of a frequent disease, such as hypothyroidism, not only on the fatty acid, but also on the TG, phospholipids and cholesterol contents of PMN cells, key effectors of the innate immune response. Thus, taking into account above observations and considering that PTU has been used in experimental models to cause hypothyroidism [4, 9], we were aimed to investigate (i) the expression of mRNAs of key factors involved in lipid metabolism and (ii) the fatty acyl composition of total lipids from PMN cells from PTU-induced hypothyroid rats. Results from these studies could contribute to the understanding of the relationship between lipid metabolism and PMN functions.

Materials and Methods

Chemicals

Lipid standards were acquired from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from Merck Laboratory (Darmstadt, Germany).

Experimental model

Adult female Wistar rats bred in our animal facility (pathogen-free certified) were used throughout the experiments. Animal body weights were 175 ± 25 g at the onset of the treatment. The rats were kept in a light and temperature–controlled room with lights on between 06.00–20.00 h and temperature between 22–24 ºC. Rat chow (Cargill, Córdoba, Argentina) and tap water with or without PTU supplementation (0.1 g/L) were available *ad libitum*. PTU daily intake was 2.61± 0.08 mg/día/rat. After a 30-days treatment,

euthyroid (ET) and hypothyroid (HT) rats were killed by decapitation between 09.00–10.00 a.m. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Experimental protocols were reviewed and approved by the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals.

Thyroid profile

The thyroid profile was assessed by chemiluminescence's assays. Free T_4 (FT_4) and TSH were determined by using a commercial kit for the autoanalyzer ACS: 180 PLUS (Ciba Corning, Bayer Health Care, MA, USA) in the Laboratory of Clinical Analysis at the San Luis Hospital. The intra-assay coefficient of variation was 1.54 % for estimation of FT_4 at 1.42 ng/dL and 3.01 % for TSH at 18.53 µIU/mL. The ACS:180 TSH assay is a two-site immunoassay, and even though not specific for rat; it has been previously validated in our laboratory with satisfactory and reproducible results. The cross-reactivity of either FSH or LH in this assay was less than 6%.

Haematological parameters

Whole blood samples were analysed for white blood cells (WBC), red blood cells (RBC) and platelets (PLT) counts using the automated haematological analyser, Cell Dyn 1200 (Abbott Laboratories, Philippines).Smears preparations were stained by May Grünwald–Giemsa [17], and leukocytes subsets were identified by light microscopy and counted by the same operator.

Isolation of polymorphonuclear leukocytes

PMN leukocytes were isolated by density-gradient centrifugation with Ficoll-Histopaque 1083 solution [18]. Blood was drawn into a tube containing EDTA and sedimented on 3 % dextran for 30 min. Supernatant was recovered and spooned at 240 x g for 10 min. The pellet of PMN cells was resuspended in 8 ml of sterile saline (PBS, pH 7.40) buffer, layered onto 4 ml Ficoll-Histopaque solution and centrifuged at 400 x g for 40 min at 20 ºC. Contaminating erythrocytes were eliminated by 30 sec hypotonic lysis in sterile distilled water with the addition of 1.6 % NaCl for maintaining low osmolarity. Purified cells were finally resuspended in Hanks' balanced salt solution (HBSS, pH 7.40) without Ca^{2+} and Mg^{2+}. Cell purity was >98% and cell viability >96% as assessed by the trypan blue dye exclusion test [19].

Lipid analysis

Blood samples were centrifuged at 400 x g for 10 min at 25 ºC within 1 h of sampling, and serum was separated and kept at 4 ºC until were analyzed. TG, total cholesterol (TC), and HDL-C measurements were performed within 4 h of samples isolation. Serum lipid concentrations from ET or HT rats were determined in fresh serum samples. HDL-C was measured in the supernatant after precipitation of other lipoproteins by the dextran sulfate-$MgCl_2$ procedure [20]. LDL-C was calculated by means of the Friedewald formula [21]. TG, TC and HDL-C were measured by enzymatic methods (kits from Wiener Lab, Rosario, Argentina) using a Metrolab 2300 autoanalyzer. For calibration purpose a standard (reference) lyophilized serum from Wiener Lab was used. Total lipids were extracted from PMN leukocytes using chloroform-methanol (2:1; chromatographic purity) according to the method described in Folch et al. [22]. Lipid extracts were dissolved in n-hexane and the different lipids were separated by thin-layer chromatography with an N -hexane-diethyl ether-acetic acid (80:20:1; v/v/v) as solvent system [23]. Complex lipids were localized using iodine vapour, identified by means of their Rf values and scrapped-off the plates. Phospholipids were determined according to Fisk et al. [24] while free (FC) and esterified (EC) cholesterol were determined following the method described in Zack et al. [25] after saponification [26]. TG content was quantified by the method of Sardesai and Manning [27].

Fatty acid analysis

The fatty acyl composition of complex lipids in PMNs was determined by capillary gas-liquid chromatography (c-GLC). In brief, PBS washed cell suspensions (containing 1 x 10^6 PMNs) were treated as indicated below to obtain total lipid extracts. After dried under nitrogen at (2 ºC), the extracts were saponified and processed to obtain the fatty-acyl methyl esthers (FAMEs) as indicated in a previous paper [28], except that in this case we used a capillary column mounted on a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA) equipped with a terminal computer integrator and a data station A-680 Plus. Before injection into the c-GLC system, the FAMEs were purified by HPTLC chromatography using silica gel glass plates from Merck (Darmstadt, Germany) with zone concentration bands. The solvent system was previously detailed [23]. Chromatographic patterns of the FAMEs were identified by comparison of their relative retention times with authentic standards (Sigma Chem. Co, St. Louis, MO) running in parallel with each set of samples. Relative mass distribution of each analysis was calculated electronically by quantification of each fatty acid peak area divided by the sum of all areas.

Neutral lipid accumulation in PMN cells by Nile red staining test

The lipid content in isolated PMN cells was fluorometrically determined using Nile red, a vital lipophilic dye for labeling neutral lipid accumulation in cytosol. Stock solution of Nile red (1000 µg/mL) in acetone was prepared and stored protected from light. Isolated PMNs (1-2 x 10^6/mL) were resuspended in PBS, smeared on delipidated cover slips and fixed with 2 % paraformaldehyde for 15 min. Leukocyte smears were incubated for 10 min with Nile red solution at a final concentration of 2.5 µM in PBS at 25 °C. Cellular preparations were washed with PBS and counted, by the same operator, in an epifluorescence microscope Nikon Eclipse E50i (Nikon Corporation, Tokyo, Japan) with excitation to 494 nm and emission 521 nm. A total of 400 cells of each group were counted with a 1000X magnification.

Gen Target	Primer Forward	Primer Reverse	Product length (bp)	Gen Bank Acces
LDL-R	5′TGACGGGCTGGCGGTAGACTGGATC 3′	5′CAATCTGTCCAGTACATGAAGC 3′	180	NM175762
HMGCoAR	5′ACATCCGTCTCCAGTCCAAAA3′	5′CAGGTTTCTTGTCGGTGCAA3′	150	NM013134
SREBP-2	5′GCAAAGCCTC GTGACATCCT3′	5′GCCATTGGCTGTCTGAGTCAA3′	100	NM001033694.1
DGAT-2	5′ATGCCTGTGCTTC GGGAGTA 3′	5′ACCTCCCACCACGATGACAA3′	120	NM001012345.1
β-Actin	5′CGTGGGCCGCCCTAGGCACCA3′	5′TTGGCCTTAGGGTTCAGAGGGG3′	243	NM031144.2

Table 1. Primers sequence used in Real Time PCR

Total RNA preparation

PMN cells isolated from 10 mL of whole blood were resuspended in 300 µL HBSS and homogenized in 700 µL TRIzol reagent solution (Invitrogen, USA). Total RNA was extracted following the manufacturer's suggested protocol, except that the isopropanol precipitation step was performed overnight at -20 ºC. Purified total RNAs were then quantified and assessed for purity by measurement of the ratio 260/280 nm optical density using an UV spectrophotometer Beckman DU-640 B (CA, USA).

Reverse transcription reaction

cDNA was synthesized using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and random primer hexamers. A reaction mixture (15 µL) containing 200 ng of total RNA, 100 pmol of random primer hexamers and RNAse-free water was pre-heated at 70 °C for 5 min. Reverse transcription reaction was allowed to occur at 37 °C for 1 h after adding 5 µL of 5X reaction buffer, 20 µmol dNTPs and 200 IU of MMLV-RT in a final incubation volume of 26 µL. The mixture was then heated at 75 °C for 15 min in order to inactivate the MMLV-RT. The cDNA solution was then stored at –20 °C until use. Fragments coding for LDL-R, HMGCoAR, SREBP-2, DGAT-2 and β-actin (as endogenous control) were amplified and quantified by Real Time-PCR using specific primers. These were designed using the Primer Express 3.0 software. The forward (FW) and reverse (RV) primer sequences used for each gene are listed in Table 1.

Relative quantification of mRNA LDL receptor, HMGCoA reductase, SREBP-2 and DGAT-2 expression

The mRNA expression levels of LDL-R, HMGCoAR, SREBP-2 and DGAT-2 were quantified using real-time quantitative PCR. Twenty µL of the reaction mixture contained 12.5 µL of SYBR Green PCR master mix (Applied Biosystems, Cheshire, U.K.), 200 nM of each primer, 5.5 µL of water and 5 µL of cDNA. The thermalcycling conditions were: 10 min at 95 ºC followed by 40 cycles of 15 sec at 95 ºC - 1 min at 60 ºC and a final dissociation step (at 95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec) in an ABI PRISM 7500 real-time PCR system (Applied Biosystems). β-Actin mRNA levels were determined as endogenous control. No-template control tubes (NTC) -containing water instead of template mRNA- were run under the same reaction and thermalcycling conditions for each pair of primers. Samples were processed in triplicates and the experiment was repeated at least twice. The specificity of the amplification reaction was confirmed by examining the dissociation curve. Data were obtained with the 7500 System SDS version 1.3 software (Applied Biosystems) and were expressed as Ct (cycle threshold), ΔCt (target Ct - β-Actin Ct), and RQ (relative quantification). The background fluorescence was removed by resetting the noise band. The relation between the Ct and the initial amount of cDNA was found to be linear. The correlation coefficient (*r*) was near to 1, and PCR amplification efficiencies of the target genes and the endogenous control were similar and close to 100%. Real-time PCR efficiencies were calculated from the slopes of the standard curves according to the equation E = 10(-1/slope). RQ values were obtained using the $2^{-\Delta\Delta C_T}$ method, adjusting the target mRNA to the β-actin mRNA expression and considering the target/β-actin normalized expression in rat liver as reference RQ = 1. RQ values were expressed as mean ± standard deviation and were statistically analysed using the Graph Pad Prism 4.0 software.

HMGCoA reductase analysis by Western Blotting

After washing the PMNs suspensions with HBSS (centrifuging twice at 240 x g for 1 min with 500 µl each), cell homogenates were prepared in lysis buffer (50 mM Tris-HCl, pH 7,4 with 1 X proteases inhibitors) and centrifuged at 10300 x g for 30 min. Protein concentration in the supernatant was measured using the Lowry method. Five microliters of sample buffer (250 mM Tris, 4% β-mercaptoethanol, 4% SDS, 40% glycerol, and 0.002% bromophenol blue) were added to all samples (40 ug of protein in each) and each mix was taken to a final volume of 20 ul with distilled water. The samples were then boiled for 5 min, and applied to 10% polyacrylamide gels.

Pre-stained molecular weight markers of 4 to 250 kDa were applied to one lane (SeeBlue Plus 2 Prestained; Invitrogen/Life Technologies). The electrophoretically separated proteins were transferred to a PDVF Immobilon-P (Millipore Corporation) membrane. Each membrane was blocked with 5% nonfat dry milk and incubated at room temperature for 1 h with a 1:500 dilution of a rabbit polyclonal antiserum against rat HMGCoAR protein (H-300, Santa Cruz Biotechnology) followed by a secondary antibody conjugated to horseradish peroxidase (Thermo Scientific Pierce Antibodies). Images were acquired

Determinations	Euthyroid group (ET)	Hypothyroid group (HT)
Free T_4 (ng/ dL)	2.85 ± 0.28	0.11 ± 0.01[a]
TSH (μIU/mL)	1.14 ± 0.23	33.86 ± 5.10[a]
Triacylglycerides (mg/dL)	51.50 ± 4.11	36.13 ± 4.39[a]
Total cholesterol (mg/dL)	47.75 ± 1.92	61.50 ± 3.36 [a]
LDL-cholesterol (mg/dL)	27.45 ± 2.78	41.15 ± 3.03 [a]
HDL-cholesterol (mg/dL)	10.00 ± 1.15	12.38 ± 0.84

Table 2. Effects of PTU treatment on serum concentrations of free T_4, TSH, triacylglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol in rats. Values are means ± SEM for groups of eight rats. [a] $P < 0.05$ compared with the respective control groups using *t*-test.

Determinations	Euthyroid group (ET) n=14	Hypothyroid group (HT) n=16
Phospholipids (μg P/10^6 cells)	2.36 ± 0.44	2.04 ± 0.27
Free cholesterol (μg/10^6 cells)	4.07 ± 0.68	1.38 ± 0.43 [a]
Esterified cholesterol (μg/10^6 cells)	3.92 ± 0.66	1.61 ± 0.41 [a]
Triacylglycerides (μg/10^6 cells)	4.72 ± 0.20	12.91 ± 2.75 [a]

Table 3. Effects of PTU treatment on concentrations of phospholipids, free cholesterol, esterified cholesterol and triacylglycerides in polymorphonuclear leukocytes of rats. Values are means ± SEM; n, number of rats. [a] $P < 0.05$ compared with the respective control groups using *t*-test.

Fatty Acyl chains	Euthyroid group (ET)	Hypothyroid group (HT)
14:00	6.03 ± 0.08	8.40 ± 0.12 [b]
16:00	29.45 ±0.09	28.65 ± 0.07 [a]
18:00	25.88 ± 0.05	20.82 ± 0.08 [b]
18:1 n-9	12.98 ± 0.10	11.41 ± 0.19[a]
18:2 n-6	9.14 ± 0.09	12.74 ± 0.17 [b]
18:3 n-3	0.1000 ± 0.0001	0.10 ± 0.09
20:4 n-6	11.44 ± 0.10	10.41 ± 0.07[a]
22:6 n-3	0.07 ± 0.04	0.08 ± 0.04
16:0/16:1	13.95 ± 0.28	16.59 ± 0.26 [a]
18:0/18:1	1.75 ± 0.03	1.56 ± 0.02 [a]
18:2/20:4	0.8000 ± 0.0001	1.22 ± 0.02 [a]
Unsaturation index	82.30 ± 0.80	92.29 ± 0.45 [b]

Table 4. Effects of hypothyroidism on the relative fatty acyl composition of total lipids from polymorphonuclear leukocytes of rats. Values are means ± SEM of specific fatty acids percents. Relative mass distribution of each analysis was calculated electronically by quantification of each fatty acid peak area divided by the sum oh all areas. Analyses were performed by capillary gas-liquid chromatography using three pools (n = 3) of blood samples from either eight ET or eight PTU-treated (n = 3) rats. [a] $P < 0.01$ and [b] $P < 0.001$ compared with the control groups using *t*-test.

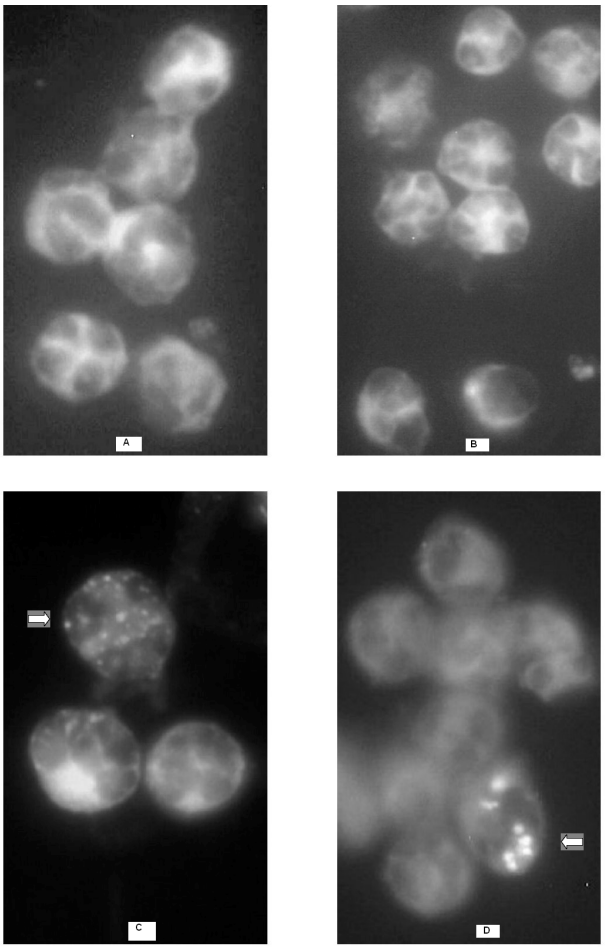

Fig. 1. Representative photomicrographs of Nile red stained lipid droplets of PMNs pools from euthyroid (A and B, n = 4) and hypothyroid (C and D, n = 4) rats. The neutral lipid accumulation was more frequent and pronounced in PMNs from hypothyroid rats (33.0 ± 5.0 % PMNs exhibited abundant lipid droplets in HT rats, while this percentage was only 7.1± 1.1 % in ET animals) as detected by neutral lipid staining. PMNs were treated with Nile red and observed with fluorescence microscopy. A total of 400 cells of each group were counted. Arrows indicate PMNs with abundant lipid droplets. Original magnification, 1000X.

after enhanced chemiluminescence (ECL) using a FluorChem HD2 imager from AlfaInnotech (San Leandro, CA). The mean of intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). The HMGCoA-R protein levels were normalized against ACTIN (endogenous control).

Statistical analysis

Statistical analysis was performed using the unpaired Student's t-test. When variances were not homogeneous, we performed unpaired t-test with Welch's correction. A statistical significance was considered valid when $P < 0.05$.

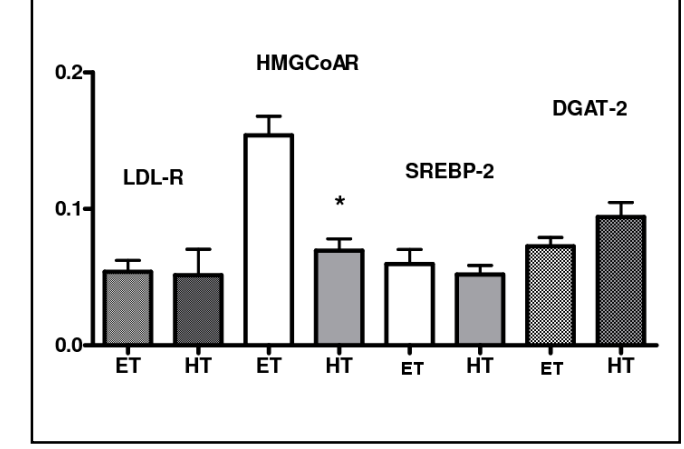

Fig. 2. Relative quantifications of LDL-R, HMGCoAR, SREBP-2 and DGAT-2 mRNA expressions in PMN leukocytes from ET and HT rats. The corresponding mRNA expression level in rat liver was used as calibrator. The quantification was done by Real Time-PCR using four pools of eight ET and PTU-treated rats, respectively. The determination was done in triplicate. No significant differences were observed between ET (left bar) and HT rats (right bar) in the LDL-R, SREBP-2 and DGAT-2 level mRNA expressions. The HMGCoAR level mRNA expression was markedly diminished in the HT group. * $P < 0.05$ compared with the respective control groups using *t*-test.

Results

Serum levels of thyroid hormones and TSH

We found the FT4 levels decreased, and that TSH concentration increased in the serum of PTU-treated (HT) rats as compared to the ET group (Table 2).

Effect of hypothyroidism on haematological parameters

Since it is known that hyperthyroidism treatment with PTU is associated with agranulocytosis and leukopenia [29, 30], we measured haematological parameters in our experimental model.

Experimental hypothyroidism did not affect the counts of WBC (ET: 5,80 x 103/mm3 ± 0,76 vs HT: 9,99 x 103/mm3 ± 1,73), RBC (ET: 5,11 x106/mm3 ± 0,11 vs HT: 5,95 x106/mm3 ± 0,41) and PLT (ET: 568,80 x 103/mm3 ± 47,70 vs HT: 374,50 x 103/mm3 ± 108,40). In our model of hypothyroidism the PMN percentage was not statistically distinguishable from control data (ET: 40,83 % ± 4,77 vs HT: 43,43 % ± 2,82).

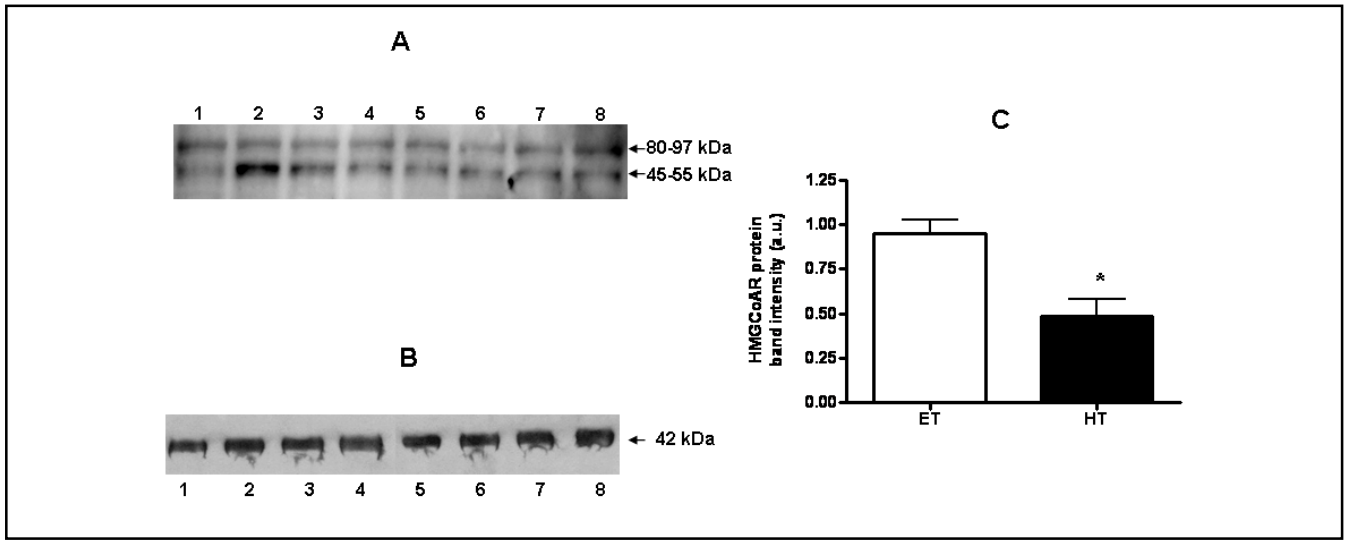

Fig. 3. Hypothyroidism modifies the HMGCoAR protein levels in PMN leukocytes. A- Western Blotting analysis of HMGCoAR and ACTIN of PMN leukocytes from euthyroid and hypothyroid rats. The major HMGCoAR band is at 80-97kDa. The lower bands are proteolytic products resulting from sample processing. Samples of PMN homogenates, 40 µg, from euthyroid (1,2,3,4) and hypothyroid (5,6,7,8) rats were applied. B – Levels of HMGCoAR protein corrected against ACTIN as endogenous control. Bars represents means ± SD (n=4). * indicates significant difference (p = 0.0078) using unpaired t test.

Effect of hypothyroidism on serum lipids

Since it is well known that thyroid dysfunction leads to changes in lipoprotein metabolism, we measured the concentrations of TG, TC, HDL-C and LDL-C in the serum of HT and ET rats. Circulating TG concentration was diminished in HT rats; however, TC and LDL-C were markedly elevated. The PTU treatment had no significant effect on circulating HDL-C levels (Table 2).

Effect of hypothyroidism on lipid levels in PMN leukocytes

Changes in the serum lipid profile observed in the HT group might influence the lipid composition in different cells and tissues, including those involved in the immune function. This led us to measure the phospholipids, FC, EC and TG contents in PMN leukocytes (Table 3). Hypothyroidism did not alter the phospholipids concentration in PMN cells; however, it modified the cholesterol levels. EC and FC concentrations decreased in the PMNs of HT rats. On other hand, the TG levels increased in the PMNs of HT animals in comparison to ET rats.

Effect of hypothyroidism on PMN leukocytes fatty acid composition

The experimental hypothyroidism also affected the relative contents of fatty acyl chains from complex lipids in PMNs cells. We observed a decreased amount of palmitic, stearic, and arachidonic acids but an increased content of myristic and linoleic acids in complex lipids in HT PMN cells. In agreement with these changes, the unsaturation index increased in the HT group as compared to ET group (Table 4).

Effect of hypothyroidism on neutral lipid accumulation in PMN cells

Lipid droplets or lipid bodies (LBs) are spherical, non–membrane-bound lipid-rich cytoplasmic inclusions found inside various inflammatory cells. The LBs in neutrophils are largely composed of TG. We found increased TG levels in PMNs. Then, we decided to investigate the LBs contents in PMN. Hypothyroidism increased the LBs accumulation in PMNs as observed by the Nile red staining. In HT rats almost 33.0 ± 5.0 % of the PMNs exhibited abundant LBs, while this percentage was only 7.1 ± 1.1 % in ET animals (Fig. 1).

Effect of PTU treatment on levels of LDL-R, HMGCoAR, SREBP-2 and DGAT-2 mRNA expression in PMN leukocytes

To elucidate the causes of the decreased cholesterol content in leukocytes, we determined the expression levels of LDL-R, involved in cholesterol uptake, and HMGCoAR, the rate-limiting enzyme of

cholesterol biosynthesis. Experimental hypothyroidism did not affect the mRNA levels of LDL-R in PMNs but it diminished significantly the HMGCoAR mRNA expression (Fig. 2).

SREBP-2 is a transcriptional factor that activates not only the LDL-R transcription but also other genes directly involved in cholesterol homeostasis. Thereafter, we quantified SREBP-2 expression in the PMNs of HT and ET rats. We found no significant differences between groups (Fig. 2).

The increase in the TG content observed in PMNs of HT rats could be due to an increase in de novo TG biosynthesis. DGAT-2 catalyzes the terminal and only committed step in the cellular TG synthesis. Thus, we decided to quantify the DGAT-2 mRNA levels in the PMN cells of HT rats. Results demonstrated there were no significant changes between groups (Fig. 2).

Effect of PTU treatment on protein level of HMGCoAR in PMN cells

Taking into account that HMGCoAR mRNA content was significantly diminished in the PMN cells of HT rats, we measured the HMGCoAR protein levels. Consistently with above results, levels of HMGCoAR protein decreased in the PMNs of HT in comparison to ET animals (Fig. 3).

Discussion

Thyroid status has a great influence on serum lipid levels, and it is also very important for the lipid content of cells [31, 32]. Maintenance of the physiological lipid composition in the phagocyte cells is essential for proper biomembrane structure and function, and for the normal functional performance of this type of immune cells [33, 34]. Here, we studied the effects of hypothyroidism on lipid levels and on lipid metabolizing enzymes expression in the PMN cells. As expected, our hypothyroid animal model was characterized by reduced FT_4 and increased TSH serum levels (Table 2). These results are consistent with the FT_4 and TSH levels observed by other authors [4, 35, 36] in a similar animal model.

Regarding circulating lipid levels, our results (Table 2) are consistent with others' previous findings which also showed changes in serum lipid composition hallmarked by increased TC and LDL-C contents in HT individuals [3, 34, 37]. However, the HDL-C was not altered in our experimental model. Hypothyroidism also provoked a significant decrease of the serum TG concentration. The inverse correlation between serum TC and thyroid hormone levels has been known from experimental observations which date back to over 70 years [38]. Hypothyroidism-associated hypercholesterolemia would mainly be due to a decreased rate of receptor-mediated catabolism of the plasmatic lipoproteins in the liver. On the other hand, the decrease in the TG levels has been attributed to an overall increase in the peripheral lipoprotein lipase activity [39].

In this study, we also found hypothyroidism caused a significant decrease of FC and EC in the PMN cells (Table 3). Results strongly suggest that these alterations were likely due to a decrease in the mRNA and protein levels of HMGCoAR, the key enzyme for cholesterol biosynthesis. Previous studies [40] showed that hypothyroidism causes a decrease in the mRNA, protein and activity levels of HMGCoAR in the rat liver. These effects were reverted by a T_3 treatment [40]. On the contrary, Cachefo and collaborators [41] found an increased HMGCoAR mRNA expression in mononuclear leukocytes from hyperthyroid patients; however, they did not find significant changes in LDL-R expression [41]. We did not find significant changes in LDL-R expression in PMNs of our hypothyroid rats, either. Thus, the mRNA expression of LDL-R may not be regulated by the thyroid hormone, at least in this type of immune cells. Moreover, other experimental data suggest that cholesterol synthesis and LDL-R-mediated cholesterol uptake may work independently, and that receptor-mediated LDL uptake is a secondary compensatory mechanism following cholesterol synthesis [14]. However, other authors found that HMGCoAR as well as LDL-R mRNA, protein levels and activity, were all increased in the liver of rats treated with T_3 or thyromimetics [42]. It is possible that in our model, LDL-R activity is affected without changes in its mRNA levels as seen by Kuo et al. [43]. They demonstrated that changes in plasma-membrane fatty acyl composition of the human hepatocarcinoma cell line HepG2 can alter LDL uptake and metabolism without changing gene expression. Probably, the increase of the unsaturated fatty acid in the PMNs of the HT rats could impinge on the LDL-C uptake by LDL-R without modifying LDL-R mRNA levels. On the other hand, Salter et al. [6] demonstrated that hepatocytes

isolated from PTU-treated rats showed a significant decrease in cholesterol esterification. This finding agrees with the decreased content of EC in PMNs that we found in HT rats.

The sterol regulatory element-binding proteins (SREBPs) are transcriptional factors regulated by cholesterol levels [44]. Shin et al [45] showed that SREBP-2 promoter is activated by the thyroid hormone. They postulated that changes in the LDL-R mRNA expression in the mice liver occur as a consequence of changes in SREBP-2 levels caused by thyroid hormone alterations. However, we did not find any change in the SREBP-2 mRNA levels in the PMNs from PTU-induced HT rats.

As shown in Table 3, TG content increased in PMNs isolated from the HT rats. The lipid droplets or LBs are cytoplasmic inclusions present within neutrophilic [46], eosinophilic [47] and basophilic leukocytes [48], and many other cell types [49]. Results obtained from neutrophils suggest that LBs are composed largely of TG [50]. On the other hand, LBs from PMNs become more prominent in number and size when these leukocytes are engaged in inflammatory responses [46]. In our experiments, we observed that cellular TG concentration increases in the PMNs of HT along with arise in the number and size of LBs within those cells, as corroborated by microscopic observation after Nile red staining (Fig. 1).

Interestingly, TG pools within LBs do not function as physiological stores of arachidonic acid for immediate leucotriene generation [51]. The identification of TG lipase and its activator CGI-58 within LBs opens the perspective that this arachidonate could be used as a storage pool to replenish (upon transfer) lipid body arachidonyl-phospholipids. Thus, the activation of cytosolic phospholipase A_2 would provide arachidonate for local eicosanoid biosynthesis from this endogenous pool [52]. Although our results showed that the percentage of arachidonic acid in hypothyroid PMNs decreased significantly, it is possible that its levels increases within the LBs. This would have important physiological consequences [52], that should be further investigated.

It is well known that DGAT (AcylCoA:diacylglycerol acyltransferase) is a key enzyme in the pathway of TG biosyntesis. In human tissues, the highest DGAT-2 mRNA expression is observed in liver, mammary gland, adipose tissue, testis, and peripheral leukocytes; with lower expression in adrenal, thyroid, and brain [53]. We did not find significant changes in the DGAT-2 mRNA levels in PMNs of HT rats; however, we can not discard posttranslational and/or posttranslational effects of thyroid hormone alterations. It is well known that DGAT activity is acutely regulated by phosphorylation-dephosphorylation mechanisms [54], and that the sn-3 position in TG contains mostly unsaturated fatty acyl chains [55]. Other studies showed that DGAT is more active with C18:1 and C18:2 both acting as enzyme substrates [56]. PMNs isolated from HT rats have a great increase of the C18:2 content. Thus, such increase in the substrate concentration could stimulate the enzymatic activity in our experimental model.

As stated previously, the main alteration found in fatty acyl profile was a misbalance between saturated and unsaturated fatty acyl chains, which lead to an increased unsaturation index. Results obtained from hypothyroid men showed a decrease in 16:0, 18:0 contents in plasma, whereas the levels of all the monounsaturated fatty acids increased in the erythrocytes [57]. Van Doormaal and collaborators [57] also found an increased proportion of 18:2 n-6 contents, facts that completely agree with our results. The increased 18:2 n-6/20:4 n-6 ratio observed by us is consistent with the alteration in the $\Delta 6$ desaturase activity observed by those authors in hypothyroid men.

The modifications in the pattern of fatty acid observed in PMN leukocytes from HT rats are similar to those observed by others in the rat liver [58]. In PTU-induced hypothyroidism, all the enzyme activities involved in the biosynthesis of fatty acids (acetyl-CoA carboxylase, fatty acid synthetase and microsomal chain elongation and desaturation reactions) are strongly reduced after three days of drug administration [59].

Our results demonstrated that hypothyroidism causes important changes in the lipid composition of PMN cells. Lipid rafts are cholesterol and sphingolipid-enriched domains of cellular membranes [60]. Interactions between cholesterol, phospholipids and sphingolipids present in rafts in specific amounts, create highly "lipid-ordered" microdomains [61-63] which recruit signaling proteins and act as staging areas for molecular signaling complexes. Cholesterol bioavailability regulates membrane raft composition and the trafficking of transient receptor potential channel (TRPC) proteins. These proteins form cation channels that can mediate agonist-induced Ca^{2+} entry [64]. Many facets of PMNs activation depend directly on Ca^{2+} entry into the cell [65], thus the

cellular cholesterol deficiency could limit inflammatory responses and regulate the immune cell activity. Recently, non-genomic actions of thyroid hormones have been described [66]. Some functions of PMNs, including generation of reactive oxygen species (ROS), are affected by hypothyroidism and are mediated by a membrane-bound receptor for thyroid hormones [67, 68]. The lipid alterations observed in this work, may affect the membrane composition and, consequently, the no-genomic effects of thyroid hormones on PMN cells.

In addition, previous evidence demonstrated that changes in cellular cholesterol modify diverse PMN functions. In neutrophils, the cholesterol depletion increases lightly the adhesion efficiency to P-selectin–coated beads, increase rolling velocity while decrease the firm arrest to activated endothelium [69]. In conclusion, we suggest that the PMNs lipid alterations observed in HT rats could modify the physiology of these cells, and therefore, the host defense mechanism(s).

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