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THYRONINES AND PROBUCOL INHIBITION OF HUMAN CAPILLARY ENDOTHELIAL CELL-INDUCED LOW DENSITY LIPOPROTEIN OXIDATION

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Abstract—Oxidized lipoproteins have been implicated as important factors in the pathogenicity of atherosclerosis. Thus, antioxidants play a significant role in inhibiting a critical step in atheroma progression. Previously, we demonstrated that thyronine analogs inhibit Cu^{2+}-induced low density lipoprotein (LDL) oxidation. In the present study, we examined the effect of thyronine analogs on endothelial cell (EC)-induced LDL oxidation. LDL was incubated with or without EC in the presence or absence of various concentrations of thyronine, vitamin C, or probucol at 37° in a humidified atmosphere (95% air, 5% CO_2). Thyronine analogs, probucol, and vitamin C inhibited EC-induced LDL oxidation in a concentration-dependent manner. The concentration of each agent (μM) producing 50% inhibition (IC_{50}) of EC-induced LDL oxidation for thiobarbituric acid reactive substances (TBARS) and electrophoretic mobility, respectively, was as follows: 0.294 and 0.417 for levo-thyroxine (L-T_4); 0.200 and 0.299 for L-triiodothyronine (L-T_3); 0.125 and 0.264 for dextro-thyroxine (D-T_4); 0.203 and 0.304 for reversed triiodothyronine (rT_3); 1.02 and 1.44 for probucol; and 13.6 and 14.9 for vitamin C. Thyroid binding globulin (TBG) inhibited EC-induced LDL oxidation; further, thyronines bound to TBG exhibited more antioxidant activity than unbound thyronines. Pretreatment of EC with any of the thyronines decreased the ability of EC to oxidize LDL. Also, our results showed that a synergistic interaction exists between vitamin C and T_4 in the inhibition of EC-induced LDL oxidation. The T_4 and TBG concentrations that inhibited LDL oxidation were in the physiological range. We conclude that T_4, like the pharmacological agent probucol, reduces oxidative modification of LDL and thus may act as a natural inhibitor of atherogenesis.

Key words: antioxidants; ascorbic acid; endothelium, vascular; lipid peroxidation; low density lipoprotein (LDL); probucol; thyroid binding proteins, thyronines; thyroxine, D-T_4 (dextro-thyroxine); L-T_4 (levo-thyroxine); DL-T_4 (racemic thyroxine)

Modifications of LDL† may induce atheroma formation [1] through reaction with monocyte-derived macrophages [2]. Although such macrophages do not accumulate substantial amounts of cholesterol even when incubated with high LDL concentrations, they do accumulate cholesterol esters when incubated with oxLDL [3]. Since oxLDL occurs in rabbit and human atherosclerotic lesions [4, 5], measurement of operative factors in LDL oxidation may aid in the clinical assessment of disease progression. *In vivo*, the arterial tree contains modified LDL autoantibodies [6]. Furthermore, LDL eluted from atherosclerotic lesions have the same characteristics as oxLDL, and treatment of WHHL rabbits with probucol retards the development of atherosclerotic lesions [5]. OxLDL may promote atherosclerosis through (1) recognition and rapid uptake into macrophages via specific receptors with foam cell formation [7], (2) cytotoxicity [8], (3) monocyte chemotaxis induction [9], (4) tissue macrophage motility inhibition [9], (5) blockade of coronary artery relaxation by inhibiting endothelium factor [10–14], (6) promotion of platelet aggregation [15], and (7) increased monocyte adhesion to endothelial cells [16]. Since oxLDL produces atheroma, compounds possessing biologically relevant redox potentials may block this process. For example, clinically probucol not only reduces plasma cholesterol concentrations more than 10% [17], but also inhibits LDL oxidation [18]. Similarly, T_4 also exhibits a biologically relevant redox potential *in vitro* and inhibits Cu^{2+}-catalyzed LDL oxidation [19]. Experimentally, LDL incubation with EC [20] produces oxLDL. In the present study, we examined the effect of this hormone on EC-induced LDL oxidation.

The rise in the LDL cholesterol/HDL cholesterol ratio in hypothyroid patients corresponds with increased risk for coronary heart disease [21]. LDL-cholesterol elevation in myxedemic patients [22–24] putatively derives from production of oxLDL, which, in part, enhances atherosclerotic risk. Hypothyroidism treatment by L-T_4 and L-T_3 lowers LDL-cholesterol and concomitantly may reduce atherosclerosis [22–25]. Probucol, through its antioxidant action, inhibits LDL oxidation induced by cupric sulfate or endothelial cells [18]. In the present work, we examined the comparative concentration-dependent inhibition of EC-induced LDL oxidation by the thyronine compounds T_3, rT_3, D-T_4, L-T_4, and DL-T_4; additionally, we evaluated the interactions between T_4 and ascorbic acid during inhibition of EC-induced LDL

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†Abbreviations: EC, endothelial cells; ECGS, endothelial cell growth supplement; HDL, high density lipoproteins; LDL, low density lipoproteins; MDA, malondialdehybis(dimethyl acetol). oxLDL, oxidized LDL; RPE, retinal pigment epithelium; rT_3, reversed triiodothyronine; T_3, triiodothyronine, L-T_3, levo-T_3; T_4, thyroxine (tetraiodothyronine); L-T_4, levo-T_4; D-T_4, dextro-T_4; DL-T_4, racemic T_4; TBARS, thiobarbituric acid reactive substances; and TBG, thyroid binding globulin.

oxidation. Preliminary reports of this work have appeared [26].

MATERIALS AND METHODS

Materials

The Aldrich Chemical Co. (Milwaukee, WI) provided both MDA and thiobarbituric acid. American Scientific Products (Obetz, OH) supplied universal agarose film. Ultracentrifuge tubes came from Seton (Sunnyvale, CA). The Fisher Scientific Co. (Cincinnati, OH) supplied dialysis membranes (12,000–15,000 Da cutoff). The Ohio State University Cell Culture Laboratory provided PBS, M 199 medium, and human capillary EC. Sigma (St. Louis, MO) supplied thyronine analogs, sodium ascorbate, BSA and TBG. Merrell-Dow (Cincinnati, OH) donated probucol.

LDL isolation

To isolate LDL, we collected human blood samples (50 mL) in plastic tubes containing EDTA (1 mg/mL), mixed them by inversion and kept them at 4° for 3 hr. Plasma was separated by centrifugation (1000 *g*) for 20 min at room temperature. Gentamicin sulfate (1 mg/25 mL) was added to the plasma sample, and LDL (d 1.019–1.063) was isolated by sequential isopycnic ultracentrifugations [27]. LDL was dialyzed for 16–20 hr against 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.01% EDTA. Protein concentrations of LDL were measured by the method of Lowry *et al.* [28], as modified by Markwell *et al.* [29]. The purity of LDL was assessed by finding only one band when staining electrophoretograms for lipids (Fat Red 7B).

EC isolation

To isolate choroid EC, we obtained EC from human eyes.* First, excess muscle and fat were removed from the eyes, and then after making a circumferential incision, the anterior chamber and retina were removed. Finally, the RPE layer was removed by incubation with 2.5% dispase at 37° for 30 min. After washing with PBS to remove the RPE layer, the choroid was teased away from the sclera and briefly minced with crossed blades, followed by digestion in a buffer consisting of PBS with 1 mg/mL BSA and 0.5 mg/mL of collagenase type I and II for 30–60 min at 37°. The digest was centrifuged and the pellet resuspended in M 199 medium containing 15 mM HEPES, 90 μg/mL heparin, 150 μ/mL ECGS and 10% fetal bovine serum. Then the mixture was plated onto fibronectin-coated tissue culture dishes and incubated at 37° in humidified 95% air, 5% CO_2 [30].

Agent preparation and general incubation conditions

Reagents were prepared as previously described [19]. LDL (0.2 mg protein) was incubated with Hams F-10 in a total volume of 2 mL in an atmosphere of humidified 95% air, 5% CO_2 at 37°.

* The eyes were obtained from an established donor program of The Lions Eye Bank of the Lions Foundation for Eye Research.

Effect of thyronine analogs and probucol on EC-induced LDL oxidation

LDL was incubated in the presence or absence of EC [14] with or without various concentrations of thyronine analogs or probucol. Samples taken at 6 and 24 hr were stored with 1 mM EDTA at −80°. Since probucol was dissolved in ethanol, a parallel control was used. The degree of oxidation was assessed by measuring changes in electrophoretic mobility and production of TBARS.

Kinetics of the antioxidant activity of L-T_4 and probucol on EC-catalyzed LDL oxidation

LDL was incubated in the presence or absence of EC with or without various concentrations of L-T_4 (0.1–3 μM) or probucol (1–5 μM). Samples taken at 0, 3, 6, 9, 12 and 24 hr were stored with 1 mM EDTA at −80°. The degree of oxidation was assessed by measuring both TBARS and conjugated diene concentration.

EC-catalyzed LDL oxidation in the presence of TBG

LDL was incubated in the presence or absence of EC with or without TBG. Samples taken at 0, 3, 6, 9, 12 and 24 hr were stored with 1 mM EDTA at −80°. The degree of oxidation was assessed by measuring TBARS and conjugated dienes. Since TBG was in a Tris buffer containing sodium azide, a parallel control was run with this buffer. This buffer increased the oxidation of LDL by EC.

TBG effect on the antioxidant activity of thyronines in the EC-catalyzed LDL oxidation system

L-T_4 or L-T_3 (6 nmol of either one) was incubated with or without 2.1 nmol of TBG at room temperature for 12 hr. We estimated the percentage of bound L-T_4 or L-T_3 in the incubation mixture to be 24.15%. LDL was incubated in the presence or absence of EC with or without various concentrations of L-T_4 or L-T_3 preincubated with or without TBG. Samples taken at 0, 3, 6, 9, 12 and 24 hr were stored with 1 mM EDTA at −80°. The degree of oxidation was assessed by measuring TBARS and conjugated dienes.

Effects of pretreatment of EC with thryonines on their ability to oxidize LDL

EC were incubated in M 199 medium with or without 10 μM L-T_4 or L-T_3 for 24 hr at 37° in an atmosphere of humidified 95% air, 5% CO_2. After incubation, EC were washed three times with PBS and two times with Hams F-10. Then LDL was incubated in the presence or absence of EC with or without pretreatment with thyronines. Samples were taken at 0, 2, 4, 6, 8, 10 and 24 hr and stored with 1 mM EDTA at −80°. TBARS and conjugated dienes concentration were used to assay the degree of LDL oxidation.

Effect of interaction between L-T_4 and ascorbate on EC-induced LDL oxidation

LDL was incubated in the presence or absence of EC [9] with or without 0.2 μM L-T_4 in the presence or absence of various concentrations of ascorbic acid (1–20 μM). Samples taken at 0, 2, 4, 6, 8, 10 and 24 hr were stored with 1 mM EDTA at −80°. The degree of oxidation was assessed by measuring both TBARS and conjugated dienes concentration.

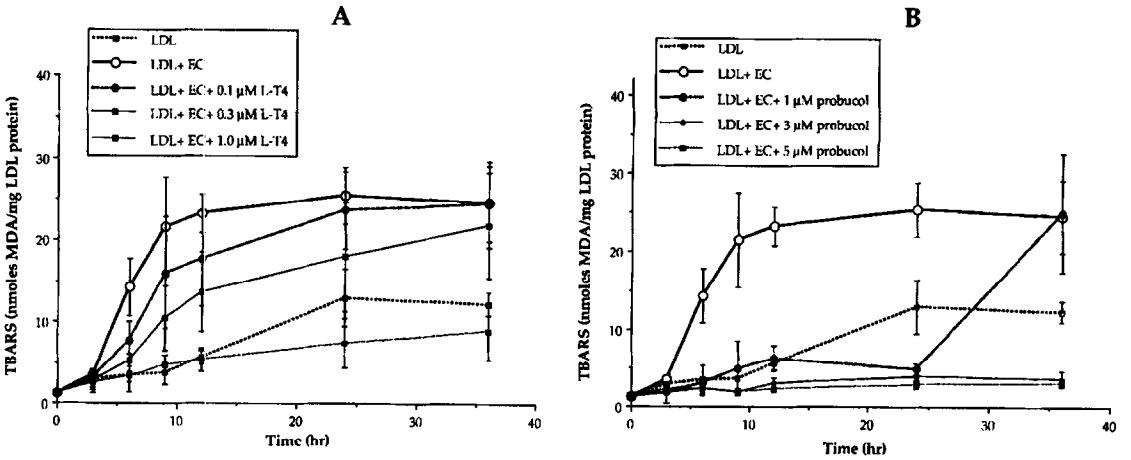

Fig. 1. Kinetics of the antioxidant activity of L-T_4 (panel A) and probucol (panel B) on EC-induced LDL oxidation as assessed by measuring TBARS. LDL (200 μg LDL protein) was incubated with or without EC in the presence or absence of various concentrations of L-T_4 or probucol in 95% air, 5% CO_2 at 37°. Samples were taken at 0, 3, 6, 9, 12, 24 and 36 hr and stored with 1 mM EDTA at −80°. All values are means ± SD, N = 3 different subjects.

TBARS measurement

Aliquots (0.5 mL) of the incubation mixture, containing 50 μg LDL protein, were mixed with 20% trichloroacetic acid (1.5 mL). Thiobarbituric acid (1.5 mL of a 0.67% solution in 0.05 M NaOH) was added with mixing, and the reaction mixture was incubated in a water bath (80–90°) for 45 min. Samples were centrifuged at (2000 *g*) for 10 min, and the fluorescence of the supernatant was measured by using excitation and emission wavelengths of 510 and 553 nm, respectively [31]. TBARS were calculated from an MDA standard curve prepared from MDA. Results are expressed as nanomoles MDA per milligram LDL protein.

Electrophoretic mobility measurement

Electrophoresis of LDL was performed on agarose gels in barbital buffer, pH 8.6, at 90 V for 35 min. Gels were dried for 30 min in an oven (Corning) and then stained with Fat Red 7B dye for 10 min. Excess stain was removed by washing in methanol:water (2:1, v/v), the gels were oven dried, and the stained bands were scanned (Corning 720 densitometer). To determine electrophoretic mobility, the distance from the origin to the center of the β-lipoprotein peak was measured on the scanned electrophoretogram [32]. LDL and oxLDL migrated maximally at 11 and 33 mm, respectively.

Diene conjugation measurement

Diene conjugation was determined spectrophotometrically at 234 nm. The amount of diene conjugation was based on a molar absorbance of 2.95×10^4 [33].

Statistical analyses

ANOVA was used to determine if there were any differences with this experimental design among the agents by employing the appropriate statistical treatment with repeated measures performed for each of the LDL oxidation parameters. Each of the agents was compared with probucol, using a post-hoc Scheffe F-test if $P < 0.05$ [34]. Simple regression analysis was utilized to test the validity of all concentration–responses.

RESULTS

Exposure of LDL to EC induced LDL oxidation and produced 1.2-, 5.1-, 7.6-, 8.2- and 8.8-fold increases in TBARS after 3, 6, 9, 12 and 24 hr of incubation, respectively. Incubation of LDL with EC in the presence of various concentrations of L-T_4 (Fig. 1A) or probucol (Fig. 1B) inhibited LDL oxidation. At all incubation times, the TBARS concentration varied inversely with the L-T_4 and probucol concentrations, indicating a concentration-dependent inhibitory effect.

Other thyronine analogs (D-T_4, L-T_3 and rT_3) also inhibited the EC-mediated LDL oxidation in a concentration-dependent manner, as assessed by TBARS levels and electrophoretic mobility (data not shown).

The data used to plot concentration-dependent inhibition curves were employed to compute the antioxidant concentrations of thyronines and probucol for 50% inhibition (IC_{50}) of LDL oxidation. Thyronines exhibited significantly greater potency ($P < 0.05$) than probucol in inhibiting EC-induced LDL oxidation, as assessed by measuring TBARS and electrophoretic mobility (Table 1).

Table 1. Antioxidant potency of probucol and thyronines in inhibiting EC-induced LDL oxidation

Agent	IC_{50}* (μM) TBARS	Electrophoretic mobility
L-T_4	0.294 ± 0.19	0.417 ± 0.06
L-T_3	0.200 ± 0.03	0.299 ± 0.07
D-T_4	0.125 ± 0.01	0.264 ± 0.03
rT_3	0.203 ± 0.03	0.304 ± 0.10
Probucol	1.02 ± 0.03†	1.44 ± 0.52†

* LDL (200 μg) was incubated with or without EC in the presence or absence of various agent concentrations in 95% air, 5% CO_2 for 24 hr at 37°. Values are means ± SEM, N = 3 different subjects.

† $P < 0.05$ vs thyronines.

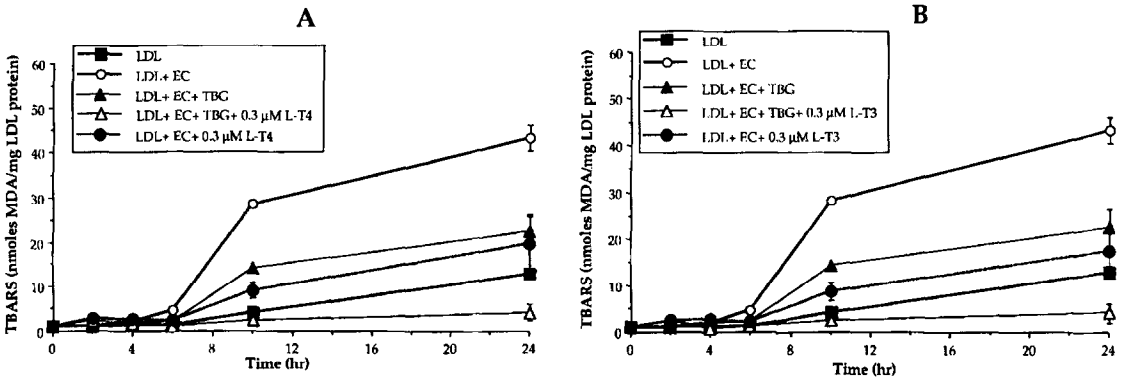

Fig. 2. Effect of thyroid binding globulin (TBG) on the antioxidant activity of L-T_4 (panel A) and L-T_3 (panel B) on EC-induced LDL oxidation as measured by TBARS. LDL (200 μg protein) was incubated with or without EC in the presence or absence of 5 μg TBG with or without 0.3 μM L-T_4 or 0.3 μM L-T_3 preincubated with or without TBG in humidified 95% air, 5% CO_2 at 37°. Samples were taken at 0, 2, 4, 6, 10 and 24 hr. All values are means ± SD, N = 3 different subjects.

Since TBG provides the principal L-T_4 and L-T_3 binding in human serum, the effects of this globulin on the antioxidant activities of L-T_4 and L-T_3 were examined. Based on TBARS measurements, TBG alone inhibited the EC-induced LDL oxidation. The inhibition of EC-induced LDL oxidation by TBG-bound L-T_4 (Fig. 2A) exceeded that produced by either L-T_4 or TBG alone. This observation also held for TBG-bound L-T_3 (Fig. 2B).

Based on TBARS, EC pretreated with thyronines had a reduced ability to oxidize LDL. Pretreatment of EC with thyronines, compared with untreated controls, produced a prolongation of the lag phase in TBARS generated by incubating LDL with EC (Fig. 3).

Vitamin C inhibited EC-induced LDL oxidation in a concentration-dependent manner (Fig. 4). The IC_{50} (μM) values were 13.6 ± 0.6 and 14.9 ± 0.6 for TBARS and electrophoretic mobility, respectively. The percentage inhibition of TBARS and conjugated dienes concentrations in the presence or absence of 0.2 μM L-T_4 plus various concentrations of ascorbic acid exceeded the expected theoretical percentage* inhibition of EC-induced LDL oxidation (Fig. 5).

DISCUSSION

These findings with EC demonstrated that L-T_4, in the physiological concentration range, inhibited LDL oxidation. TBG did not interfere with L-T_4 inhibition of LDL oxidation in either study. Further, even without added L-T_4. TBG inhibited LDL oxidation. In addition, this study confirms the work of others that EC have the capacity to oxidize LDL, as demonstrated by the increase in TBARS, electrophoretic mobility, and conjugated dienes [31, 35].

L-T_4 and probucol inhibited cell-induced LDL oxidation in a concentration-dependent manner as evidenced by reduced TBARS and conjugated dienes. Each agent also produced an increase in the resistance of LDL to EC-induced LDL oxidation by an increased lag before oxidation. We demonstrated the physiological importance of L-T_4 as a natural antioxidant since L-T_4, at concentrations equal to or below physiological concentrations, inhibited EC-induced LDL oxidation. Thus, L-T_4 may serve as one of the natural defenses against atherosclerosis.

We also demonstrated that all of the thyronine analogs inhibited EC-induced LDL oxidation. Since these thyronine analogs possess biologically relevant redox potentials and exhibit no enantioselective antioxidant activity, enzyme-mediated reactions in this process appear unlikely.

Direct scavenging of the free radicals provides the most likely mechanism for thyronine blockade of LDL oxidation. L-T_4 may act as an antioxidant wherein free radicals like peroxyl or hydroxyl radicals abstract a phenolic hydrogen from T_4 to form phenoxyl radicals. Thus,

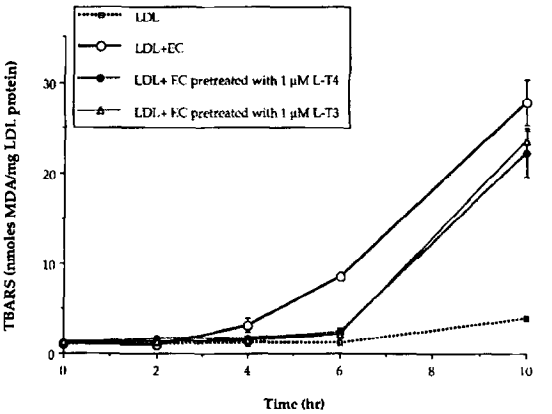

Fig. 3. Effect of pretreatment of EC with thyronines on the ability of EC to oxidize LDL as assessed by measuring TBARS. EC were incubated in M 199 medium with or without 1 μM L-T_4 or L-T_3 for 24 hr at 37° in an atmosphere of humidified 95% air, 5% CO_2. After incubation, EC were washed three times with PBS and two times with Hams F-10. LDL (0.2 mg protein) was then incubated with Hams F-10 in a total volume of 2 mL in an atmosphere of humidified 95% air, 5% CO_2 at 37° in the presence or absence of EC with or without thyronine pretreatment. Samples were taken at 0, 2, 4, 6, 10 and 24 hr (data for 24 hr are not shown), and stored with 1 mM EDTA at −80°. All values are means ± SD, N = 3 different subjects.

* The theoretical percentage inhibition is computed by adding the actual percentage inhibition values obtained when either L-T_4 or ascorbic acid was incubated alone.

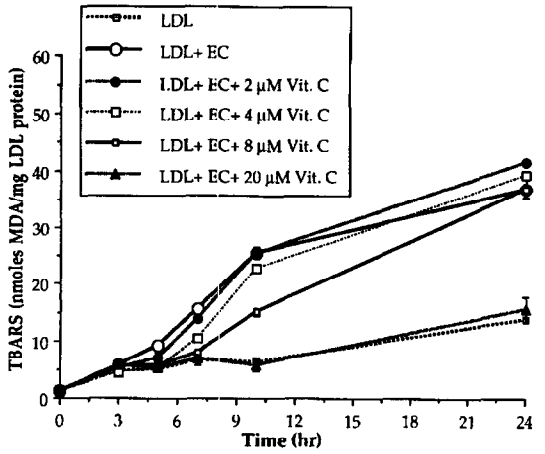

Fig. 4. Kinetics of the antioxidant activity of vitamin C on EC-induced LDL oxidation as assessed by measuring TBARS. LDL (200 μg LDL protein) was incubated with or without EC in the presence or absence of various concentrations of vitamin C in 95% air, 5% CO_2 at 37°. Samples were taken at 0, 3, 5, 7, 10 and 24 hr, and stored with 1 mM EDTA at –80°. All values are means ± SD, N = 3 different subjects.

unstable radicals may rearrange to form the more stable 1,4-hydroquinone and 3,5-diiodotyrosine. Alternatively, or in combination with this scavenging mechanism, L-T_4 may inhibit EC-induced LDL oxidation by blocking lipoxygenase and phospholipase A_2 activities. The activity of these cellular enzymes may play important roles in EC-induced production of free radicals [36, 37].

In human plasma, T_4-binding globulins, T_4-binding prealbumin, and serum albumin represent the primary transport system for thyroid hormones [38]. However, a small percentage of both L-T_3 and L-T_4 binds to plasma lipoproteins [39]. This lipoprotein-bound T_4 may inhibit LDL oxidation.

Based on TBARS and conjugated dienes, TBG, the major thyroid binding protein, inhibited EC-induced LDL oxidation. TBG antioxidant activity could be due to: (1) thiol group-induced free radical scavenging, (2) chelation of metal ions, or (3) increasing the steady-state concentration of naturally existing antioxidants. Our observation that TBG inhibited the EC-induced LDL oxidation both alone and when it was bound to L-T_4 strengthens the likelihood of a physiological antioxidant role for TBG. Further, the TBG-bound L-T_4 antioxidant potency exceeded that of either TBG or L-T_4 alone.

L-T_4 or L-T_3-pretreated EC had decreased ability to oxidize LDL, possibly because the L-T_4 or L-T_3: (1) adsorbs onto the cell surface subsequent to washing and thus traps free radicals generated by EC before attacking LDL particles; (2) enters cells and inhibits cellular mechanisms for generating free radicals; and (3) is stored and then released by cells into the extracellular medium to protect LDL against EC-induced LDL oxidation.

We have confirmed the antioxidant effect of ascorbate in inhibiting EC-catalyzed LDL oxidation in a concentration-dependent manner, as assessed by TBARS and conjugated dienes [40–42]. Further, ascorbate acts synergistically with L-T_4 to inhibit EC-induced LDL oxidation, an effect that may be attributable to T_4 recycling through ascorbate reduction of the L-T_4 phenoxyl radical formed during cell-generated free radical scavenging.

The results reported here extend our previous observations about the capability of thyronines to reduce atherogenicity through inhibition of Cu^{2+}-catalyzed LDL oxidation [19]. Further, these agents may also protect LDL from oxidation by increasing apoA-I-containing lipoproteins [43–45]. *In vivo*, thyroid hormones may play a role in preventing LDL oxidation since lipoproteins isolated from hypothyroid rats show an increased propensity for oxidation.*

* Olubadewo JO, Robinson TJ, Wingard MA, Lee N, James PA and Ochillo RF, Hypothyroidism alters lipoprotein metabolism and peroxidation in a way that enhances lipid deposition in arterial walls. In: *9th International Symposium on Atherosclerosis 1991*, p. 186.

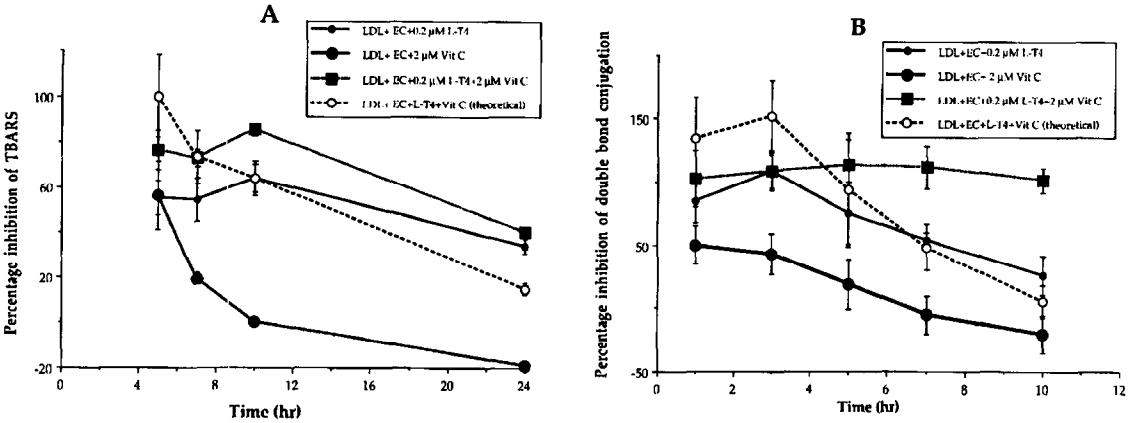

Fig. 5. Inhibition of EC-induced LDL oxidation by thyroxine, vitamin C, and thyroxine in combination with vitamin C as assessed by measuring TBARS (panel A) and conjugated dienes (panel B). LDL (200 μg protein) was incubated with or without EC in the presence or absence of 0.2 μM L-T_4 and/or 2 μM vitamin C in 95% air, 5% CO_2 at 37°. The concentrations of TBARS at 5, 7, 10 and 24 hr, respectively, were: 5.07 ± 0.53, 6.64 ± 0.86, 6.27 ± 0.64 and 13.95 ± 0.51 nmol/mg LDL protein for LDL alone; 5.12 ± 0.30, 15.60 ± 0.71, 25.49 ± 1.00 and 37.01 ± 1.63 nmol/mg LDL protein for LDL incubated with EC. The concentrations of conjugated dienes at 1, 3, 5, 7 and 10 hr, respectively, were: 0.106 ± 0.009, 0.147 ± 0.005, 0.192 ± 0.004, 0.250 ± 0.014 and 0.295 ± 0.002 μmol/mg LDL protein for LDL alone; 0.133 ± 0.01, 0.240 ± 0.036, 0.366 ± 0.035, 0.435 ± 0.034 and 0.554 ± 0.007 μmol/mg LDL protein for LDL incubated with EC. Samples were stored with 1 mM EDTA at –80°. All values are means ± SD, N = 3 different subjects.

Conversely, elevated rat T_4 induces lipid peroxidation through increases in liver microsomal oxidative capacity [46]. Also, thyroid hormones in the presence of H_2O_2 initiate lipid peroxidation of isolated erythrocytes [47]. Thus, altering the balance between antioxidant and redox cycling-initiated oxidation by T_4, a likely characteristic of the resting state of the biological system, may profoundly affect lipoprotein metabolism and atherosclerosis. These studies, coupled with our previous observations [19], may provide insights for the physiological role of these hormones in lipid and lipoprotein metabolism and their interaction with vitamin C and TBG.

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