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INHIBITION OF LOW DENSITY LIPOPROTEIN OXIDATION BY THYRONINES AND PROBUCOL

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Abstract—Oxidation of low density lipoproteins (LDL) results in increased macrophage uptake of LDL which may contribute to the formation of macrophage-derived foam cells in the early atherosclerotic lesion. In this study we show that thyroxine (T_4), its optical antipodes, certain desiodo analogs and probucol inhibited cupric sulfate-catalyzed oxidation of human LDL in a concentration-dependent manner as assessed by measuring the electrophoretic mobility, thiobarbituric acid reactive substances (TBARS) and LDL degradation in mouse macrophages. In Cu^{2+}-catalyzed LDL oxidation at 24 hr, the TBARS level was 80 nmol/mg LDL protein/24-hr incubation. The concentrations (μM) of each agent producing 50% inhibition in the formation of oxidized LDL (IC_{50}) for TBARS, electrophoretic mobility and macrophage degradation, respectively, were 1.13, 1.27 and 1.30 for reversed triiodothyronine; 1.33, 1.80 and 1.27 for triiodothyronine; 1.33, 1.37 and 1.37 for racemic thyroxine, DL-T_4; 1.10, 1.40 and 1.50 for L-T_4; 1.13, 1.33 and 1.23 for D-T_4; and 1.47, 1.63 and 1.37 for probucol. No differences in inhibitory potency were observed when rT_3, T_3, the optical antipodes of T_4 and the hydrophobic antioxidant drug probucol were compared. In air-induced LDL oxidation, TBARS was 16.1 nmol/mg LDL protein/6-hr incubation. The IC_{50} concentrations (μM) for TBARS and diene conjugation, respectively, were 0.187 and 0.336 for D-T_4; 0.205 and 0.243 for L-T_4 and 1.30 and 3.02 for probucol. With air-induced LDL oxidation conditions, the L-T_4 concentrations included the physiological range, and thyroid-binding globulin did not modify the inhibitory effect of the endogenous enantiomer, L-T_4. Putative uptake of this stereoisomer into LDL inhibited oxidation of these lipoproteins. Since concentrations of these thyronines which blocked air-induced LDL oxidation were in the physiological range, we conclude that thyronines, like the pharmacological agent probucol, limit the oxidative modification of LDL and thus may serve as natural inhibitors of atherogenesis.

Serum low density lipoproteins (LDL)†-cholesterol increases the risk of coronary heart disease [1]. However, extensive atheroma occurs in the arterial tree even in the absence of a functional LDL receptor to mediate uptake in homozygous familial hypercholesterolemia [2]. Thus, there exists, in the arterial wall, an alternate pathway for LDL uptake. Large numbers of lipid-laden foam cells found in the subendothelial space of atherosclerotic arteries originate from monocyte-derived macrophages [3]. These macrophages do not accumulate substantial amounts of cholesterol even when incubated with LDL in high concentrations but do accumulate considerable amounts of cholesterol when incubated with oxidized LDL (oxLDL) [4]. OxLDL occurs in both rabbit and human atherosclerotic lesions [5–7], and techniques employed to evaluate factors influencing LDL oxidation may be useful in the clinical assessment of the disease progression. Further, *in vivo* within the arterial tree, LDL modification is demonstrated by autoantibodies which recognize modified LDL and other proteins altered by lipid oxidation products (e.g. malondialdehyde) [8]. OxLDL may promote atherosclerosis through: (a) uptake through scavenger receptors with accumulation of cholesterol esters [4], (b) endothelial cell cytotoxicity induced by free radical lipid peroxidation [9, 10], (c) increased recruitment of monocytes into intima by chemoattractant action [11, 12], (d) retention of macrophages in the intima by inhibiting motility [12], and (e) vasoconstriction of coronary arteries by impairing endothelium-dependent relaxation [13].

Many compounds with diverse chemical structures have biologically relevant redox potentials, lower LDL cholesterol serum concentrations, and consequently may be antiatherogenic. Probucol is one such agent which, in clinical studies, reduces plasma cholesterol concentrations by over 10% [14], but also inhibits LDL oxidation [15]. Like probucol, thyroxine (T_4) also exhibits a biologically relevant redox potential *in vitro*‡ and could serve as an endogenous protective agent. Now to evaluate this hypothesis we have studied this hormone and, comparatively, several related compounds for their ability to inhibit LDL oxidation. Experimentally,

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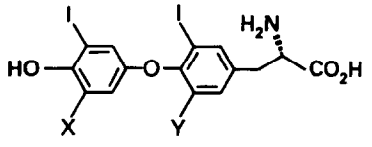
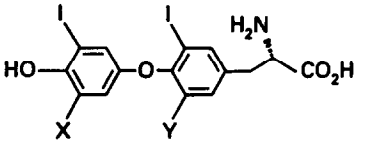
† Abbreviations: LDL, low density lipoproteins; oxLDL, oxidized LDL; T_3, triiodothyronine; T_4 thyroxine; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde bis(dimethyl acetal); PBS, phosphate-buffered saline; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; and TBG, thyroid binding globulin.

‡ McCreery RL, personal communication. Cited with permission.



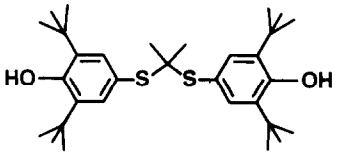
Triiodothyronine (D-T_3), X = H, Y = I

D-Thyroxine (D-T_4), X = Y = I



Reverse triiodothyronine (R-T_3), X = I, Y = H

L-Thyroxine (L-T_4), X = Y = I



Probucol

Fig. 1. Chemical structures of thyronines and probucol.

LDL oxidation can be achieved by incubation with cupric sulfate [16], endothelial cells, arterial smooth muscle cells [17] and human monocytes [18]. Cupric sulfate-catalyzed oxidation of LDL facilitates generation of thiobarbituric acid reactive substances (TBARS) and increases LDL electrophoretic mobility, presumably due to lipid peroxidation and neutralization of the positively charged epsilon amino groups on lysine residues of LDL apoB [19]. LDL oxidation mediated by cupric ion results in a marked decrease in LDL amino group reactivity which corresponds with increased LDL uptake and degradation via either acetyl-LDL or oxLDL receptors [20].

The rise in the LDL cholesterol/HDL cholesterol ratio in hypothyroidism increases the risk for coronary heart disease [21, 22]. LDL-cholesterol elevation in myxedemic patients may be a function of several mechanisms including increased cholesterol synthesis and absorption [23], decreased hepatic lipase and lipoprotein lipase activities [24], and defects in receptor-mediated catabolism of LDL [25]. Such elevations in LDL may be accompanied by the production of oxLDL and, in part, be responsible for enhanced atherosclerotic risk in such individuals. Treatment of hypothyroidism by L-T_4 and triiodothyronine (T_3) lowers LDL-cholesterol and additionally may reduce atherosclerosis [23–26]. Probucol, through its antioxidant action, inhibits LDL oxidation induced by cupric sulfate or endothelial cells [15]. The comparative concentration-dependent inhibition of cupric sulfate-catalyzed LDL oxidation by thyronine compounds (T_3, rT_3, D-T_4, L-T_4, and DL-T_4) (Fig. 1) with probucol as a control are described in this article. To simulate conditions which may occur *in vivo*, selected compounds were investigated as inhibitors of air-induced LDL oxidation. The degree of oxLDL production was determined by measuring TBARS, electrophoretic mobility, diene conjugation and degradation by macrophages. Preliminary reports of this work have appeared [27, 28].

MATERIALS AND METHODS

Materials. Malondialdehyde bis(dimethyl acetal) (MDA) and thiobarbituric acid were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Universal agarose film was obtained from American Scientific Products (Obetz, OH). Ultracentrifuge tubes were purchased from Seton (Sunnyvale, CA). Dialysis membranes (12,000–15,000 Da cutoff) were purchased from the Fisher Scientific Co. (Cincinnati, OH). Phosphate-buffered saline (PBS), minimal essential medium (MEM), fetal bovine serum and serum free Dulbecco's modified Eagle's medium (DMEM) were obtained from The Ohio State University Cell Culture Laboratory. Thyronine analogs, bovine serum albumin (BSA), and thyroid binding globulin (TBG) were purchased from Sigma (St. Louis, MO), and probucol was a gift from Merrell–Dow (Cincinnati, OH). [^{125}I] was purchased from Amersham (Arlington, IL).

LDL isolation. Human blood samples (50 mL) were collected in plastic tubes containing EDTA (1 mg/mL), mixed by inversion, and kept at 4° for 3 hr. Plasma was separated by centrifugation (2000 *g*) for 20 min at room temperature. Gentamicin sulfate (1 mg/25 mL) was added to the plasma sample. LDL (d 1.019 to 1.063) was isolated by sequential isopycnic ultracentrifugations [29]. 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.* [30] as modified by Markwell *et al.* [31]. Purity of LDL was assessed by the finding of only one band when electrophoretograms were stained for either lipids (Fat Red 7B) or proteins (Amido Black).

Agent preparations. Thyronine analogs (T_3, rT_3,

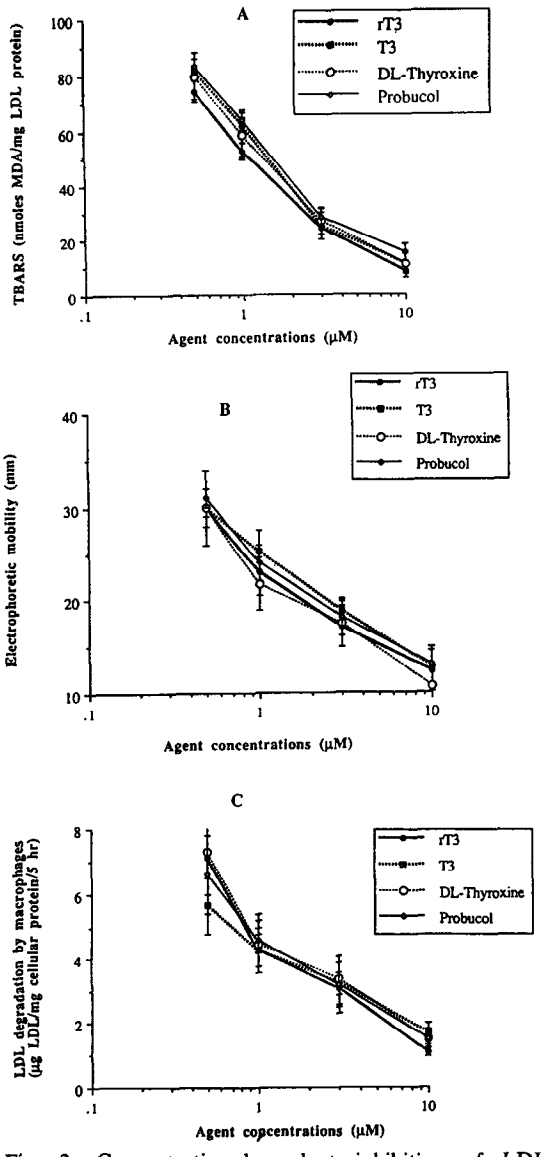


Fig. 2. Concentration-dependent inhibition of LDL oxidation parameters by thyronine analogs and probucol. Concentration responses are: Panel A, TBARS; Panel B, electrophoretic mobility, and Panel C, LDL degradation by macrophages. LDL (200 μg LDL protein) was incubated with 10 μM $CuSO_4$ in the presence or absence of various concentrations of rT_3, T_3, DL-T_4 or probucol in 95% air, 5% CO_2 for 24 hr at 37°. All values are means ± SD, N = 3 different subjects.

L-T_4, D-T_4, DL-T_4; Fig. 1) were dissolved in 0.01 M phosphate buffer, pH 9.5, and the solution was adjusted to pH 7.4. Probucol was dissolved in ethanol.

Iodination of LDL. LDL was labeled with [^{125}I] by a modification of the iodide monochloride method of McFarlane [32], by using glycine-NaOH buffer, pH 9.5. Iodide monochloride was added to LDL protein in a molar ratio of 10:1 in the presence of carrier free [^{125}I]. The unbound iodide was removed by extensive dialysis against 0.1 M Tris buffer, 0.01% EDTA, 0.85% NaCl, pH 7.4. After dialysis, about 98% of [^{125}I] was precipitable with trichloroacetic acid (15%, v/v); about 2% remained unbound. The iodination procedure was 25.5% efficient. Prior to use, ^{125}I-labeled LDL was sterilized by passage through a Millipore filter (0.22 μm) [33, 34].

Incubation mixture: Cu^{2+}-catalyzed LDL oxidation. [^{125}I]LDL (0.2 mg protein) was incubated with Ham's F-10 in a total volume of 2 mL in an atmosphere of humidified 95% air, 5% CO_2 for 24 hr at 37° in the presence or absence of cupric sulfate (10 μM) [12] with or without various concentrations of thyronine analogs or probucol. Since probucol was dissolved in ethanol and added to the incubation mixture at a final concentration of 2%, v/v, an equivalent alcohol control was included. Ethanol alone did not modify LDL oxidation. The degree of oxidation was assessed by measuring changes in electrophoretic mobility, production of TBARS, and LDL degradation by macrophages.

Incubation mixture: Air-induced LDL oxidation. LDL (0.2 mg protein) was incubated as described previously with the following modifications: cupric sulfate was not added; the total incubation time was 6 hr; and when samples were withdrawn for analysis, EDTA was added to give a final concentration of 1 mM in order to chelate trace amounts of transitional metal ions. The degree of oxidation was assessed by measuring both TBARS and diene conjugation.

Incubation mixture: Assessment of the role of chelation in the antioxidant activity of thyronines. LDL (0.2 mg protein) was incubated with Ham's 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 cupric sulfate (3 or 5 μM) with or without 2 μM L-T_3, L-T_4 or probucol. Samples were taken at 0, 4, 10 and 24 hr. The degree of oxidation was assessed by measuring TBARS.

Incubation mixture: Kinetic experiment. LDL (0.2 mg protein) was incubated with Ham's 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 cupric sulfate (5 μM) with or without 1 μM L-T_3, L-T_4 or probucol. Samples were taken at 0, 1, 2, 3, 4, 12 and 24 hr. The degree of oxidation was assessed by measuring TBARS.

Measurement of TBARS. 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 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 (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 [35]. TBARS concentrations in samples were calculated from an MDA standard curve prepared from MDA. Results are expressed as nanomoles MDA per milligram of LDL protein.

Measurement of electrophoretic mobility. Electrophoresis of LDL was carried out 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

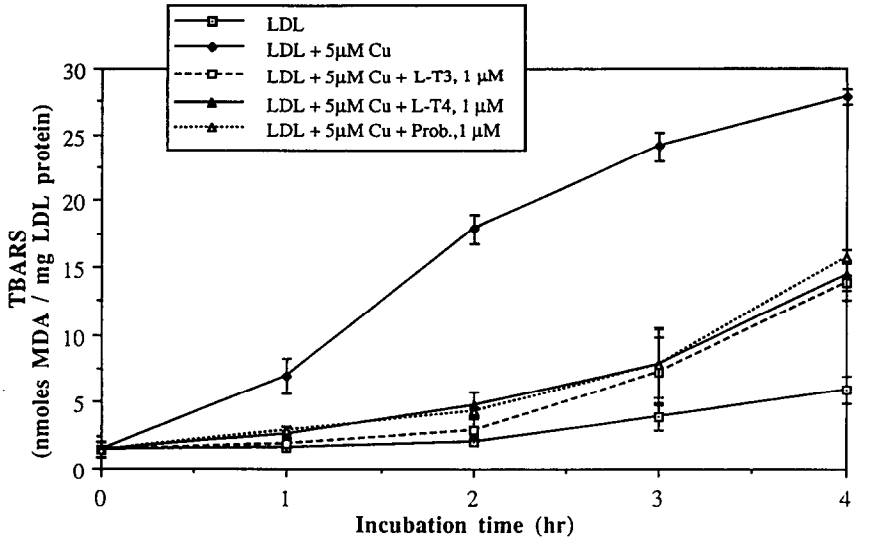


Fig. 3. Effects of thyronine analogs and probucol on both the lag and propagation phases of the Cu^{2+}-catalyzed LDL oxidation parameter, TBARS. LDL (200 μg LDL protein) was incubated with or without 5 μM $CuSO_4$ in the presence or absence of 1 μM L-T_4, L-T_3 or probucol in 95% air, 5% CO_2 for 24 hr at 37°. Samples were taken at 0, 1, 2, 3, 4, 12 and 24 hr (data for 12 and 24 hr are not shown). All values are means ± SD, N = 3 different subjects.

was removed by washing in methanol:water (2:1, v/v). The gels were again oven-dried, and stained bands were scanned (Corning 720 densitometer). Electrophoretic mobility was determined by measuring the distance from the origin to the center of the β lipoprotein peak on the scanned electrophoretogram [36]. Maximal migration of LDL and oxLDL were 11 and 33 mm, respectively.

Peritoneal macrophage isolation. Resident peritoneal macrophages were isolated from ether-anesthetized female Swiss Webster mice by making a small incision to lavage the peritoneal cavity with 5 mL of PBS, pH 7.4, performing intestinal massage with a glass rod, and removing the peritoneal washings with a plastic syringe. The washing process was repeated three times. Washings were combined and centrifuged (500 *g*) for 5 min, and the cell-free supernatant was decanted from the pellet. The cell pellet was vortexed gently and suspended in 5 mL MEM containing 10% fetal bovine serum. Viable cells were determined by the Trypan Blue exclusion method. Aliquots of the cell suspension (containing 4×10^6 cells) were transferred to 60 mm Petri dishes containing 3 mL of the MEM with 10% fetal bovine serum and incubated at 37°. After a 2-hr incubation, non-adherent cells were removed, and the remaining cells were cultured for 24 hr in 3 mL of MEM under 95% air, 5% CO_2 at 37° [37].

LDL degradation by macrophages. After culturing for 24 hr, cells were washed with 5 mL of serum-free DMEM. DMEM (2.7 mL) was added together with 0.3 mL of [^{125}I]LDL (containing 30 μg LDL protein) previously incubated with cupric sulfate and various concentrations of drugs. Petri dishes were incubated at 37° for 5 hr in a humidified atmosphere of 95% air, 5% CO_2. After incubation, aliquots of the medium (0.5 mL) were removed and 0.5 mL of 2% BSA was added as a carrier. Then 1 M KI (250 μL) was added followed by 250 μL of 50% trichloroacetic acid. The mixture was centrifuged, and 0.5 mL of the supernatant was treated with 250 μL of 5% silver nitrate to precipitate the free iodide. The radioactivity remaining in the supernatant was counted as a measure of LDL degradation and expressed as micrograms LDL per milligram of cellular protein per 5 hr [38, 39].

LDL oxidation in the presence of thyroid-binding globulin. LDL was subjected to air-induced LDL oxidation conditions in the presence or absence of various concentrations of L-T_4 preincubated with or without 4 μg/mL TBG at room temperature for 12 hr. With the assumption that 0.7 mol of L-T_4 binds to 1 mol of TBG, the estimated percentage of bound L-T_4 was 16.1% at a concentration of 300 nM T_4.

Agent pretreatment of LDL prior to oxidation. Isolated LDL was incubated in Ham's F-10 medium with 10 μM probucol, or thyronines for 24 hr at 37°. Each LDL preparation was reisolated by ultracentrifugation and dialyzed three times for 1 hr against 100× volume of 0.154 M phosphate buffer, pH 7.4. LDL was subjected to air-induced LDL oxidation conditions. Samples were taken at 0 and 4 hr and stored with 1 mM EDTA at −80°. TBARS were used to assay the degree of oxidation.

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 [40].

Statistical analyses. With this experimental design the appropriate statistical treatment was an ANOVA with repeated measures performed for each of the LDL oxidation parameters to determine if there were any differences among the agents employed. If $P < 0.05$, a post-hoc Scheffe F-test was used to

Table 1. Assessment of interference of agents with the assays for LDL oxidation*

Treatment	TBARS (nmol MDA/mg LDL protein)	Electrophoretic mobility (mm)	LDL degradation (μg LDL/5 hr/mg cellular protein)
LDL	7.5 ± 1.0	11 ± 1.0	1.3 ± 0.4
LDL + 10 μM $CuSO_4$	80 ± 2.0	33 ± 1.0	7.3 ± 0.9
LDL + 10 μM $CuSO_4$			
+10 μM rT_3	80 ± 1.0	32 ± 2.3	6.8 ± 0.7
+10 μM T_3	82 ± 3.0	33 ± 1.1	7.5 ± 0.5
+10 μM DL-T_4	80 ± 2.5	34 ± 1.5	7.5 ± 0.6
+10 μM D-T_4	79 ± 1.7	33 ± 2.3	7.1 ± 0.2
+10 μM L-T_4	81 ± 2.1	32 ± 2.0	7.1 ± 0.5
+10 μM probucol	80 ± 1.0	32 ± 1.0	7.4 ± 0.3

* LDL (200 μg protein) was incubated with 10 μM $CuSO_4$ in 95% air, 5% CO_2 for 24 hr at 37°. At the end of incubation, thyronine analogs or probucol were added followed by measuring the TBARS, electrophoretic mobility and degradation of LDL by macrophages. Values are means ± SD, N = 3 different subjects.

Table 2. Concentration-dependent effects of thyronines and probucol as inhibitors of TBARS, electrophoretic mobility and macrophage degradation of LDL

Agent	IC_{50}^* (μM)		
	TBARS	Electrophoretic mobility	LDL degradation
rT_3	1.13 ± 0.05	1.27 ± 0.15	1.30 ± 0.30
T_3	1.33 ± 0.06	1.80 ± 0.52	1.27 ± 0.65
DL-T_4	1.33 ± 0.31	1.37 ± 0.31	1.37 ± 0.35
D-T_4	1.13 ± 0.15	1.33 ± 0.25	1.23 ± 0.47
L-T_4	1.10 ± 0.10	1.40 ± 0.44	1.50 ± 0.69
Probucol	1.47 ± 0.15	1.63 ± 0.59	1.37 ± 0.49

* Data are means ± SD, N = 3 different subjects. Incubation conditions were the same as described in the legend of Fig. 2.

compare each of the agents to probucol [41]. All dose–responses were tested for significance and correlation by simple regression analysis.

Experimental design: Cu^{2+}-catalyzed LDL oxidation. Preparations of LDL from each of three different subjects (protein = 200 μg) were incubated under the following conditions: (1) with cupric sulfate (10 μM, control representing LDL oxidation without inhibitors); (2) with no cupric sulfate (baseline analyte level for quantification of IC_{50} values); (3) with cupric sulfate (10 μM) and simultaneous addition of various concentrations of thyronines or probucol for determination of concentration–response relationship and IC_{50} values; and (4) with cupric sulfate (10 μM) and addition of various concentrations of thyronines or probucol at the end of the incubation to assess inhibitor interference with TBARS, electrophoretic mobility, and LDL degradation measurements.

Experimental design: Air-induced LDL oxidation. Preparations of LDL from each of three different subjects (protein = 200 μg) were incubated under the following conditions: (1) without inhibitors (control representing LDL oxidation without inhibitors); and (2) with various concentrations of inhibitors for determination of dose–response and IC_{50}. LDL TBARS and diene values prior to incubation served as baselines for quantification of IC_{50} values.

RESULTS

Cu^{2+}-catalyzed LDL oxidation produced an 11-fold increase in TBARS, a 3-fold increase in electrophoretic mobility and a 6-fold increase in uptake by macrophages when compared to controls without cupric sulfate (Table 1). No significant differences ($P < 0.05$) could be demonstrated in TBARS, electrophoretic mobility or macrophage uptake of Cu^{2+}-treated LDL when thyronine analogs or probucol were added at the end of the incubation and compared to controls without these agents. Thus, these agents do not interfere with the TBARS, electrophoretic mobility or the macrophage-LDL degradation assays used for assessment of LDL oxidation (Table 1).

T_3, rT_3, DL-T_4 and probucol demonstrated a concentration-dependent inhibition of the production

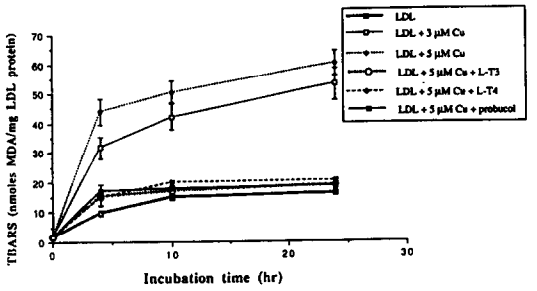


Fig. 4. Effects of thyronine analogs on the time course increase in the Cu^{2+}-catalyzed LDL oxidation parameter, TBARS. LDL (200 μg LDL protein) was incubated with or without $CuSO_4$ in the presence or absence of 2 μM L-T_4, L-T_3 or probucol in 95% air, 5% CO_2 for 24 hr at 37°. All values are means ± SD, N = 3 different subjects.

Table 3. Comparison of D-T_4, L-T_4 and probucol concentrations for 50% inhibition (IC_{50}) on oxLDL TBARS and diene conjugation

Agent	IC_{50}* (μM)	
	TBARS	Diene conjugation
D-T_4	0.187 ± 0.025	0.336 ± 0.198
L-T_4	0.205 ± 0.026	0.243 ± 0.086
Probucol	1.30 ± 0.70†	3.02 ± 2.34†

* Values are means ± SE, N = 3 different subjects. Incubation conditions were the same as described in the legend of Fig. 5.

† The IC_{50} of probucol was significantly higher ($P \leq 0.05$) than that of either L- or D-T_4.

Table 4. Percent inhibition of TBARS for LDL preincubated with probucol and thyronines

Agent	% Inhibition*
D-T_4	65.5 ± 0.707
L-T_4	83.0 ± 5.66
rT_3	80.5 ± 2.12
T_3	67.0 ± 2.83
Probucol	85.0 ± 12.7

* Isolated LDL was incubated in Ham's F-10 medium with 10 μM probucol or thyronines for 24 hr at 37°. The LDL preparation of each subject was reisolated by ultracentrifugation and dialyzed for 1 hr three times against 100× volume of 0.154 M phosphate buffer, pH 7.4. LDL was subjected to air-induced oxidation conditions, and samples were taken at 0 and 4 hr and stored with 1 mM EDTA at −80°. The TBARS of LDL preparations at 0 hr were 2.72 ± 0.90, 2.38 ± 0.96, 2.52 ± 0.93, 3.02 ± 0.84 and 2.85 ± 1.00 nmol MDA/mg LDL protein for T_3, rT_3, D-T_4, L-T_4 and probucol, respectively. The TBARS of control LDL (without antioxidants) were 2.63 ± 0.84 and 7.58 ± 1.45 at 0 and 4 hr, respectively.

of oxLDL by Cu^{2+}-catalyzed LDL oxidation (Fig. 2). TBARS levels in the incubation mixture decreased with increasing concentrations of all antioxidants (Fig. 2A). Concomitant with the decrease in TBARS was a reduction in the increased electrophoretic mobility observed for oxLDL (Fig. 2B). Additionally, the increased macrophage degradation of oxLDL was inhibited by all protective compounds in a concentration-dependent manner (Fig. 2C). There was no significant difference among the IC_{50} values of these thyronine compounds or probucol with respect to TBARS, electrophoretic mobility or macrophage degradation of LDL (Table 2). Addition of 1 μM L-T_3, L-T_4 or probucol caused prolongation of the lag phase delaying the start of the propagation phase of the Cu^{2+}-mediated LDL oxidation process (Fig. 3).

Incubation of LDL with 3 or 5 μM Cu^{2+} showed a time-dependent increase in TBARS over a 24-hr incubation period. L-T_3, L-T_4 or probucol (2 μM) inhibited Cu^{2+}-catalyzed LDL oxidation at 4, 10 and 24 hr (Fig. 4).

Exposure of LDL to air-induced LDL oxidation for 6 hr alone produced a 1.6-fold increase in TBARS (15.8/9.8) and a 1.5-fold increase in diene conjugation (0.28/0.19). Incubation with increasing concentrations of D-T_4, L-T_4, or probucol produced a concentration-dependent inhibition of LDL oxidation as assessed by TBARS and diene conjugation (Fig. 5, A and B), and the resulting data were employed to compute the IC_{50} values. The IC_{50} values for both D- and L-T_4 were significantly lower than the IC_{50} for probucol (Table 3). Based upon the TBARS data, incubation of LDL under air-induced LDL oxidation conditions following LDL preincubation with the antioxidants inhibited LDL oxidation (Table 4).

Studies involving air-induced LDL oxidation in the presence of various concentrations of L-T_4 for 6 hr inhibited the oxidation of this lipoprotein in both the presence and absence of TBG. This observation held for both TBARS and conjugated diene assays; IC_{50} values (means ± SEM, N = 3) were, respectively, 0.220 ± 0.083 and 0.268 ± 0.011 μM in the absence and 0.210 ± 0.022 and 0.216 ± 0.069 μM in the presence of TBG. However, there were significant reductions in both TBARS and conjugated dienes when TBG was present in the incubation mixture prior to induction of LDL oxidation (Table 5).

DISCUSSION

The present studies confirm that probucol added to LDL in cupric sulfate-supplemented medium inhibits the increased production of TBARS, the increased electrophoretic mobility of the oxidized lipoproteins and the oxLDL degradation by macrophages [15, 42]. In addition, our results demonstrate that thyronines blocked cupric ion-mediated increases in these parameters by a non-stereoselective process. L-T_3, L-T_4 or probucol at a concentration of 1 μM inhibited Cu^{2+}-mediated LDL oxidation over a 24-hr incubation period and caused prolongation of lag phase and delay of propagation phase of LDL oxidation. The level of TBARS

Table 5. Effect of TBG on LDL oxidation*

Incubation time (hr)	Without TBG		With TBG	
	TBARS	Diene conjugation	TBARS	Diene conjugation
0	6.13 ± 0.311	0.350 ± 0.725	6.22 ± 0.589	0.349 ± 0.089
3	8.56 ± 1.05†	0.536 ± 0.082†	7.34 ± 1.18	0.503 ± 0.058
6	14.82 ± 2.74†	0.597 ± 0.091†	11.6 ± 1.95	0.530 ± 0.074

* LDL was subjected to air-induced LDL oxidation conditions in the presence or absence of various concentrations of L-thyroxine preincubated with or without 4 μg/mL TBG at room temperature for 12 hr. Data are means ± SD, N = 3 different subjects. Values for TBARS are expressed in nmol MDA/mg LDL protein; values for diene conjugation are in μmol/mg LDL protein.

† Significantly higher than with TBG ($P \leq 0.05$).

produced by incubation of LDL with 5 μM cupric sulfate in the presence of 2 μM T_3, T_4 or probucol was lower than that produced by incubating LDL with 3 μM cupric sulfate without the agents. If the effects of thyronines or probucol were due to chelation, the TBARS level produced by incubating LDL with 5 μM cupric sulfate in the presence of 2 μM T_3, T_4 or probucol would be similar or higher than that produced by incubating LDL with 3 μM cupric sulfate (based on the assumption that 1 molecule of Cu^{2+} would be chelated by 1 or 2 molecules of thyroxine). However, our observations were not in accord with this mechanism. Thus, the inhibitory effects of thyronines on the Cu^{2+}-mediated LDL oxidation are likely to be dependent upon the redox chemistry of thyronines wherein both inhibition of free radical chain reactions and scavenging of activated oxygen species prevent oxidation of both lipids and proteins in LDL.

The role of thyroxine in preventing atherosclerosis is thought to involve increases in LDL catabolism [25], lipoprotein lipase activity [24] and the receptor binding for native LDL on skin fibroblasts [26]. Our results demonstrate that T_4 may also serve as an endogenous or exogenous anti-atherogenic agent via inhibition of free radical-mediated oxidation of LDL. Since inhibitory activity is not enantioselective, these actions are most likely not attributable to enzyme-mediated effects.

Either D- or L-T_4 inhibited LDL oxidation at pharmacological concentrations wherein IC_{50} values of 1.13 and 1.10 μM, respectively, were obtained with Cu^{2+}-catalyzed LDL oxidation (TBARS =80 nmol/mg LDL protein/24-hr incubation). The IC_{50} of L-T_4 was above the physiological range (0.05 to 0.15 μM), but oxLDL concentrations also are reported to be above those found in the blood stream [43].

However, examination of these effects in the presence of limited LDL oxidation (TBARS = 2–5 nmol/mg cholesterol) may be a more relevant system for testing the physiological activity of humoral agents [44]. Under conditions which allow for reduced oxLDL (TBARS = 16.1 nmol/mg LDL protein/6-hr incubation), the IC_{50} values for D- and L-T_4 were 0.187 ± 0.025 and 0.205 ± 0.026 (SE) μM, respectively. Since the L-T_4 concentrations include the mean of the physiological values, LDL oxidation

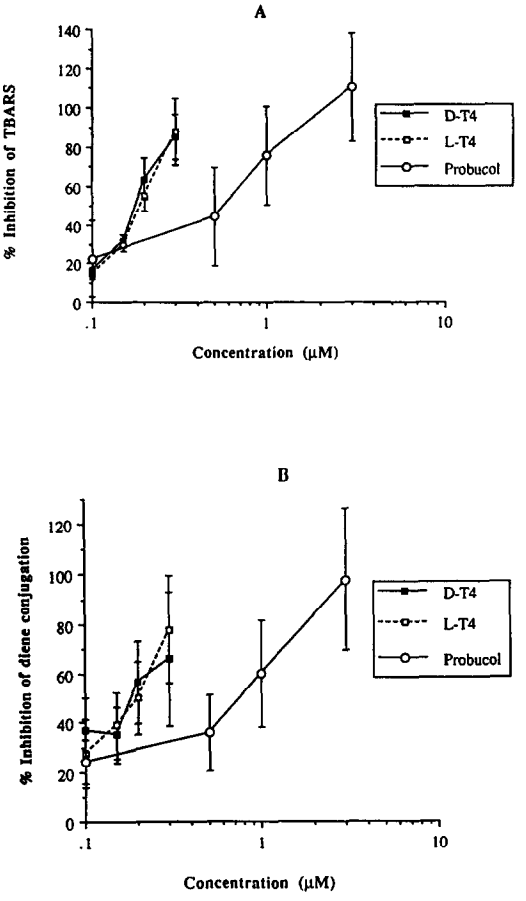


Fig. 5. Concentration-dependent inhibition of LDL oxidation parameters by thyronine analogs and probucol. Concentration responses are: Panel A, TBARS; Panel B, diene conjugation. LDL (200 μg LDL protein) was incubated in the presence or absence of various concentrations of D-T_4, L-T_4 or probucol in 95% air, 5% CO_2 for 6 hr at 37°. The TBARS and conjugated dienes of LDL incubated in the absence of agents were 15.90 ± 0.92 nmol MDA/mg LDL protein and 0.281 ± 0.054 μmol/mg LDL protein, respectively. The TBARS and conjugated diene of LDL prior to incubation (background) were 9.82 ± 1.65 nmol MDA/mg LDL protein and 0.192 ± 0.024 μmol/mg LDL protein, respectively. All values are means ± SD, N = 3 different subjects.

and its inhibition by thyroxine could take place under physiological steady-state conditions so that thyroxine may provide a natural defense against atherosclerosis.

In human plasma, thyroid hormones are transported primarily by T_4-binding globulin, T_4-binding prealbumin and serum albumin [45]. A small percentage (about 3%) of L-T_4 is bound to plasma lipoproteins with a relative distribution of VLDL, LDL and HDL of 0.8, 6.7 and 92%, respectively. T_3 binds to the same proteins with a lower affinity [46]. A T_4–LDL complex is recognized by the LDL receptor and this interaction provides an additional mode of T_4 entry into cells [47]. The T_4 binding sites on human LDL apoB-100 are distant from both the receptor binding domain and the heparin binding site of the apoprotein [48]. T_4 binds to HDL mainly through apoA-I and to a lesser extent through apoC-II/III. ApoB-100, A-I, C-II, and C-III may be responsible for T_4 binding to VLDL [49, 50]. Thus, lipoprotein-bound thyroxine could be involved, physiologically, in the protection of LDL and other lipoproteins from oxidation.

The physiological significance of L-T_4 in lipoprotein metabolism is further strengthened when it is realized that L-T_4 inhibits LDL oxidation in the presence or absence of TBG, and the latter serves as an antioxidant in serum in the unbound or LDL-bound state. Further, L-T_4 bound to TBG also may serve as an antioxidant. Further evidence that thyroxine can play this protective role for lipoproteins while putatively being bound is the finding that LDL, incubated with L-T_4 and then reisolated also protects LDL.

The work reported here extends what is known about thyronines and their ability to modify many biochemical processes involving apolipoproteins which may lead to reduced atherogenicity. Indirectly, thyroxine may protect LDL from oxidation by increasing the apoA-I-containing lipoproteins. Such lipoproteins are known to inhibit LDL oxidation [51–53]. T_4 may reduce the number of LDL particles by suppressing liver apoB-100 and increasing expression of apoB-48, liver apoA-I and apoA-IV [54–56]. An increase in the apoB-48/apoB-100 ratio at the level of mRNA editing by this hormone enhances chylomicron clearance and facilitates rapid delivery of fat-soluble antioxidants such as vitamin E to the liver. This antioxidant, in turn, may protect LDL against oxidation [54]. Also, thyroxine may serve as an antioxidant and undergo degradation while limiting free radical propagation during metallocatalytic lipid peroxidation initiated by microsomes and mitochondria [57, 58]. Further, L-T_4 protects vitamin E-deficient erythrocytes against dialuric acid-induced hemolysis [59]. Thyroid hormones at well above physiological concentrations inhibit auto-oxidation of both brain homogenates and free radical-mediated oxidation of erythrocyte membranes [60]. Reduced concentrations of thyroid hormones may play a role in LDL oxidation *in vivo* since lipoproteins isolated from hypothyroid rats show an increased propensity for oxidation.*

Conversely, hyperthyroidism in rats induces increases in the oxidative capacity of liver microsomes, and in the activities of NADH-cytochrome P450 reductase and NADPH-oxidases. These effects are accompanied by increases in lipid peroxidation [61, 62]. Also, thyroid hormones in the presence of hydrogen peroxide induce lipid peroxidation and hemolysis of isolated erythrocytes [63]. 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 have a profound effect on lipoprotein metabolism and the atherosclerotic process. These studies may provide insights concerning the extended physiological role of these hormones in lipid and lipoprotein metabolism and provide clues for the design of new drugs of clinical significance.

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