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Effects of Iodotyrosines, Thyronines, Iodothyroacetic Acids and Thyromimetic Analogues on *In Vitro* Copper-Induced Oxidation of Low-Density Lipoproteins

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ABSTRACT. We studied the effect of different thyroid compounds [(I_2, monoiodo-L-tyrosine (MIT), diiodo-L-tyrosine (DIT), L-thyronine (T_0), 3,5-diiodo-L-thyronine (T_2), 3,5,3′-triiodo-L-thyronine (T_3), 3,3′,5′-triiodo-L-thyronine (rT_3), 3,5,3′,5′-tetraiodo-L-thyronine (T_4), 3,5-diiodothyroacetic acid (TA_2), 3,5,3′-triiodothyroacetic acid (TA_3) and 3,5,3′,5′-tetraiodothyroacetic acid (TA_4)] or thyromimetics [(3,5-dimethyl-3′-isopropyl-L-thyronine (DIMIT) and 3,5-diiodo-3′-isopropyl-thyroacetic acid ($IpTA_2$)] on *in vitro* copper-induced oxidation of low-density lipoproteins (LDL). Human native LDL (0.05 g protein/L) oxidation was induced by 2.5 μmol/L of $CuCl_2$. Conjugated dienes were measured spectrophotometrically for up to 10 hr. The length of the lag phase (T_{lag}), maximum velocity of the reaction (V_{max}) and the maximum amount of generated dienes were obtained from kinetic data. T_3 increased T_{lag} and decreased V_{max} with a dependence upon concentration (0 to 3 μmol/L). There was no difference between the D_{max} obtained with Cu^{2+} alone or in the presence of the various compounds (1 μmol/L). I_2, MIT and DIT did not modify any parameter of the oxidation kinetic. T_0 and T_2 had the same antioxidant efficiency as T_3, whereas T_4 only decreased V_{max}. rT_3 increased T_{lag} less than did T_3, whereas DIMIT was the thyronine that had the most important effect. TA_2 and TA_3 were the most efficient antioxidant compounds. TA_4 decreased T_{lag} less than TA_3 did, whereas $IpTA_2$ had an effect weaker than that of the physiological acetic derivatives. The data suggest that thyroid hormones and derivatives have LDL-antioxidant properties, their importance being related to their 4′-hydroxy diphenyl ether structure and depending upon the nature and the position of substituents in this structure. BIOCHEM PHARMACOL **55**;10: 1591–1601, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. low-density lipoprotein; copper oxidation; *in vitro*; antioxidant; iodothyronines; thyromimetics

If hypercholesterolemia is well known to be a major risk factor of atherosclerosis, recent observations suggest that oxidative modification of LDL‖ increases the cholesterol atherogenicity [1, 2]. Oxidized LDL produced in the arterial intima are intensively taken by scavenger receptors on the macrophages, which directly contributes to foam cell formation. These cells, loaded with cholesteryl esters, are characteristic of the precocious lesions of the atherosclerotic plaques [3]. Moreover, oxidized LDL may affect many other aspects of arterial wall metabolism and thus contribute to the atherogenic process [4]. However, this process may be inhibited by natural or synthetic antioxidants that protect LDL against oxidative modifications [5].

Oxidation of LDL by cupric ions (Cu^{2+}) *in vitro* is a model frequently used to evaluate the protective effect of such antioxidants. This model was reported to produce oxidized LDL sharing many structural and functional properties common to LDL oxidized by cells or LDL extracted from arterial atherosclerotic plaques [6, 7]. Cu^{2+}-mediated LDL oxidation is a free radical-mediated process based on the peroxidation of LDL polyunsaturated fatty acids [8]. During the initiation phase, lipid peroxyl radicals are formed, but the propagation of the oxidation process is suppressed by endogenous antioxidants within the LDL

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‖ *Abbreviations:* apo B-100, apolipoprotein B-100; Chol, cholesterol; DIMIT, 3,5-dimethyl-3′-isopropyl-L-thyronine; DIT, diiodo-L-tyrosine; D_{max}, maximum quantity of generated dienes; HDL, high-density lipoproteins; $IpTA_2$, 3,5-diiodo-isopropylthyroacetic acid; LDL, low-density lipoproteins; MDA, malondialdehyde; MIT, monoiodo-L-tyrosine; TA_4, 3,5,3′,5′-tetraiodothyroacetic acid; TA_3, 3,5,3′-triiodothyroacetic acid; TA_2, 3,5-diiodothyroacetic acid; TBARS, thiobarbituric acid-reactive substances; T_4, thyroxine; TG, triglycerides; T_{lag}, length of the lag phase; T_3, 3,5,3′-triiodo-L-thyronine; T_2, 3,5-diiodo-L-thyronine; T_0, L-thyronine; rT_3, 3,3′,5′-triiodo-L-thyronine; V_{max}, maximum velocity of diene production.

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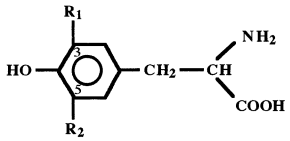
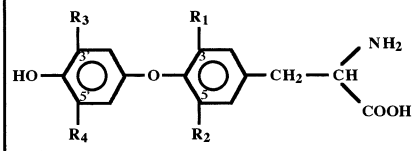
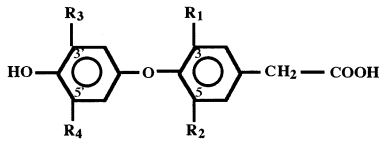
	R_1	R_2	R_3	R_4	ABBREVIATIONS
 TYROSINES	I	H	–	–	(MIT)
	I	I	–	–	(DIT)
 THYRONINES	H	H	H	H	(T_0)
	I	I	H	H	(T_2)
	I	I	I	H	(T_3)
	I	H	I	I	(rT_3)
	I	I	I	I	(T_4)
	CH_3	CH_3	$CH(CH_3)_2$	H	(DIMIT)
 ACETIC DERIVATIVES	I	I	H	H	(TA_2)
	I	I	I	H	(TA_3)
	I	I	I	I	(TA_4)
	I	I	$CH(CH_3)_2$	H	($IpTA_2$)

FIG. 1. Chemical structures of thyroid and thyromimetic compounds used in this study.

particle (tocopherols, carotenes, etc.), which results in the lag phase of oxidation [9]. When endogenous antioxidants are depleted, a rapid propagation phase occurs leading to more peroxyl radicals that are transformed into conjugated dienes by molecular rearrangement. Finally, during the decomposition phase, the dienes fragment to low molecular mass products, particularly aldehydes such as MDA. Measurement of TBARS, i.e. MDA and related compounds, is often used to evaluate the degree of LDL oxidation. However, to evaluate the three phases of the peroxidation process, it seems preferable to measure the production of conjugated dienes that absorb at 234 nm because this can be done continuously, without any extraction, is reproducible and more specific than the TBARS assay, and allows a good estimation of the lag phase and peroxidation rate [7, 10].

Alterations of thyroid metabolism may play a role in atherogenesis, because thyroid hormones are responsible for lipid abnormalities. Both primary and secondary hypothyroidisms are characterized by hypercholesterolemia associated with increased total/high-density lipoprotein (HDL) cholesterol and LDL/HDL cholesterol ratios; both the serum cholesterol and these ratios are decreased by a T_4 treatment [11, 12]. Hyperthyroid patients have lower concentrations of total cholesterol, LDL cholesterol and apo B-100 [13]. Similar alterations of lipid metabolism were reported even in subclinical hypothyroidism [14] or hyperthyroidism [15]. On the other hand, it was found that a small proportion of circulating thyroid hormones binds to lipoproteins, which may be an additional mode of T_4 entry into cells [16]. Thus, it is of interest to study the effects of thyroid hormones and thyromimetics on LDL oxidation.

At this time, only one research team has reported that T_4, T_3 and rT_3 decreased the oxidation of LDL by Cu^{2+} [17] or endothelial cells [18] *in vitro*. The authors observed that no differences existed in the antioxidant capacity of T_4, T_3 and rT_3, essentially as measured by TBARS production, electrophoretic mobility and macrophage degradation. The fact that these hormones do not have the same thyromimetic potency may lead one to believe that the observed antioxidant efficiency depends only upon their chemical structure. We wished to know: 1) if precursors, catabolites and analogues of thyroid hormones had the same effect; 2) if this antioxidant activity was similar in the different phases of the peroxidation process; and 3) what was (were) the necessary structural element(s) for this activity. Consequently, we measured the production of conjugated dienes continuously during the oxidation of LDL by Cu^{2+} in the presence of different compounds: iodotyrosines, i.e. MIT and DIT, precursors of thyroid hormones; thyronines, i.e. T_0, (T_2), T_3, rT_3 and T_4; acetic catabolites of thyroid hormones, i.e. TA_2, TA_3 and TA_4; thyromimetic analogues, i.e. DIMIT and $IpTA_2$. The chemical formulae of these compounds are presented in Fig. 1.

MATERIALS AND METHODS

Materials

Na_2HPO_4, KH_2PO_4, NaCl, KBr, $CuCl_2$, EDTA-Na_2 and chloramphenicol were obtained from Merck. BSA, $PdNO_3$, Triton X-100, Antifoam B and most of the thyroid derivatives (MIT, DIT, T_0, T_2, T_3, rT_3, T_4 and TA_3) were obtained from Sigma. TA_2, TA_4, DIMIT and $IpTA_2$ were synthesized by SmithKline Beecham. Ultracentrifuge 5-mL tubes (45248, PA, Re-Seal) were purchased from Sorvall. Dialysis membranes (SPECTRUM 12,000–15,000 Da) were obtained from Poly-Labo (Strasbourg, France).

Plasma Samples

Blood samples (~30 mL) were obtained by venipuncture from six healthy volunteers fasted overnight, i.e. three men and three women 24–52 years old, and collected in glass tubes containing 1 g/L of EDTA. After 10 min at room temperature, plasma was separated by centrifugation at 3000 *g* for 20 min at 20°. One part of the plasma was stored at −20° for further determination of cholesterol (Chol) and triglyceride (TG) concentrations. The rest was immediately treated for LDL isolation and oxidation test.

LDL Isolation

LDL (d 1.019 to 1.063) were isolated by density-gradient ultracentrifugation at 4° [19] in a Sorvall RC M120 EX ultracentrifuge (Sorvall) using KBr as density adjustment reagent. LDL-containing fraction was dialyzed in the dark for 24 or 38 hr at 4° and under nitrogen against 0.01 mol/L of phosphate buffer, pH 7.4, containing 0.15 mol/L of NaCl, 0.01% of EDTA and 1% of chloramphenicol. The buffer was first made oxygen-free by passage through a Millipore filter followed by purging with nitrogen for 1 hr, and kept for up to 3 days at 4°. The protein concentration of LDL was measured by the method of Lowry modified by Markwell *et al.* [20]. Purity of LDL preparations was checked by polyacrylamide gel electrophoresis (Lipofilm Sebia, Issy Les Moulineaux, France).

Compound Preparations

Thyroid compounds and their analogues were dissolved in 100 μL of 11 mol/L of NH_4OH that were quickly completed to 10 mL with extra-pure water. The 1 mmol/L obtained solutions, pH 9.2, were kept for up to 4 days at 4° before their further extemporaneous dissolution for the LDL oxidation test.

LDL Oxidation and Conjugated Diene Measurement

LDL oxidation was performed by the method of Esterbauer *et al.* [21], slightly modified as follows. At the end of dialysis, LDL were diluted in 0.01 mol/L of phosphate buffer, pH 7.4, without EDTA in order to obtain 0.5 g of LDL-protein per liter. Eight hundred sixty μL of the EDTA-free buffer, 20 μL of 125 μmol/L of $CuCl_2$, 20 μL of the thyroid compound dilution or of 0.01 mol/L of NH_4OH (control without compound), and 100 μL of diluted LDL were successively added in quartz cuvettes. The kinetics of LDL oxidation were determined by monitoring the change in absorbance at 234 nm at 30° against the buffer with a Uvikon 930 spectrophotometer (Kontron, Milan, Italy) equipped with a six-position automated sampler, allowing for six samples to be measured simultaneously. Absorbance was recorded every 5–6 min for 500–600 min. Final concentrations in the incubation mixture were 0.05 g of LDL-protein/L, 2.5 μmol/L of Cu^{2+} and 1 μmol/L of studied compounds, except for T_3 (tested at different concentrations [0.25–3.00 μmol/L]) and for acetic compounds (also tested at 0.25 μmol/L). The final pH of the incubation mixture was 7.4.

The experiments were performed using as fresh as possible LDL. One volunteer provided LDL, some of which was dialyzed for 24 hr and the rest for 38 hr, which made it possible to carry out two LDL oxidation tests per subject. Each LDL oxidation test systematically consisted of one cuvette with LDL alone (control of LDL particle integrity and buffer cleanliness), one cuvette with LDL and Cu^{2+} (control of oxidation), one cuvette with LDL, Cu^{2+} and T_3 (control of antioxidant capacity), and additional cuvettes with LDL, Cu^{2+} and one of the studied compounds.

The absorption data were transferred to a personal computer and then imported to the Excel spread sheet program (Microsoft) that was used for all calculations. The absorbances of each cuvette were corrected by subtracting the corresponding initial absorbance and then used to plot the diene absorbance versus time graph. The changes in absorbance/min as a function of time, i.e. the oxidation velocities, were also calculated. Three LDL oxidation parameters were considered [7]: the length of the lag phase (T_{lag}), which was determined graphically by the time intercept of the tangents to the slow and rapid increase of the kinetic profile, the maximum velocity of diene production (V_{max}), and the maximum amount of generated dienes (D_{max}). The conversion of absorbances into concentrations was based on a diene molar absorptivity of 29,500 $mol^{-1} \cdot L \cdot cm^{-1}$.

Cholesterol and Triglycerides Assays

Plasma Chol and TG concentrations were measured using cholesterol-PAP and triglycerides-PAP kits from Roche on a Cobas Bio Plus analyzer (Roche, Neuilly Sur Seine, France). The day-to-day variation coefficients of a control serum (ProBioQual, Lyon, France) were 4.1% (N = 10, mean 3.01 mmol/L) and 2.6% (N = 9, mean 0.58 mmol/L) for Chol and TG, respectively.

LDL-Cu Determination

Incubation mixtures similar to those used in LDL oxidation tests (LDL alone, LDL + Cu^{2+} and LDL + Cu^{2+} + T_3) were obtained under the same conditions. After at least 15 min at 30°, 500 μL of the mixture were filtered on a Filtron Microsep 30K column (Poly-Labo) by centrifugation at 3000 *g* and at 4° for 40 min. The remaining 500 μL of the mixture and the filtrate were immediately placed in ice and at 4° until further Cu assays. Cu was determined in buffer, in LDL alone and in filtered and nonfiltered incubation mixtures by atomic absorption spectrometry in coated graphite tubes using a SpectrAA 300 Zeeman spectrophotometer (Varian). The measurements were carried out in the presence of 1 g/L of $PdNO_3$ in samples diluted 1/10 (except for buffer and LDL alone: 1/2) with an

TABLE 1. Plasma cholesterol (Chol) and triglyceride (TG) concentrations (mmol/L) in the six volunteers fasted overnight from whom LDL were prepared

	Women			Men		
Age (y)	24	43	52	25	34	41
Chol	4.52 ± 0.22	6.20 ± 0.25	5.11 ± 0.65	4.06	5.84 ± 0.06	5.07 ± 0.20
TG	1.12 ± 0.13	0.72 ± 0.08	0.75 ± 0.07	0.66	3.89 ± 0.29	0.89 ± 0.06

Means ± SEM of 3 to 5 blood samples taken at different times, except for the 25-year-old volunteer: only one measure.

aqueous solution containing 0.1% Triton X-100 and 0.1% Antifoam B (blank solution). Calibration (0.08, 0.16 and 0.31 μmol/L of Cu) and control (0.16 μmol/L of Cu) solutions were extemporaneously obtained from a 15.8 mmol/L of Cu standard (Sigma) by successive dilutions in blank solution and were treated under the same conditions as samples. The day-to-day variation coefficient of the control was 8.2% ($N = 13$, mean 0.15 μmol/L).

Statistics

Results are presented as means ± SEM. The significant levels chosen were $P < 0.05$ (*) and $P < 0.01$ (**). Statistical analyses were performed by ANOVA using the Systat program (Deltasoft, Meylan, France). No significant differences in LDL oxidation parameters were found between the different subjects. Conversely, the length of dialysis (24 or 38 hr) modified some of these parameters, particularly T_{lag}, independently of the presence of the studied compounds. Consequently, analysis of covariance (ANCOVA) with the dialysis time as covariant was used in order to remove the variability due to the dialysis length. Relationships between continuous variables were studied by Pearson correlations and then by ANOVA based on polynomial regression models when the correlations were significant. The post hoc two-tail Dunnett test was used to determine if there were differences in parameters between each studied compound and Cu^{2+} alone. The post hoc two-tail Tukey test was then used to determine if there were any differences among active compounds. The results of LDL-oxidation tests in the presence of 0.25 μmol/L of thyroid compounds were separately analyzed by ANCOVA followed by a Tukey test.

RESULTS

Plasma Chol and TG concentrations varied between subjects and between samples in a subject, as indicated by the occasionally high SEM (Table 1). They were in the physiological ranges according to age and sex, except for the 34-year-old man who was hypertriglyceridemic. However, there was no correlation between these concentrations and the measured parameters, suggesting that the observed effects were essentially due to the studied compounds.

Figure 2 shows examples of conjugated diene absorbance kinetics obtained with LDL alone (no added Cu^{2+}), LDL + Cu^{2+} (Cu^{2+} alone) or LDL + Cu^{2+} in the presence of different T_3 concentrations. In the absence of added Cu^{2+}, absorbance slightly increased to reach a value of approximately 0.2 u. Abs. This time course of conjugated diene formation was similar to that reported when low concentrations of Cu^{2+} (<0.1 μmol/L) were added to LDL [22], indicating that some Cu^{2+} was present in buffers and/or LDL preparations (see below). In the presence of Cu^{2+} alone, absorbance kinetics revealed the three oxidation

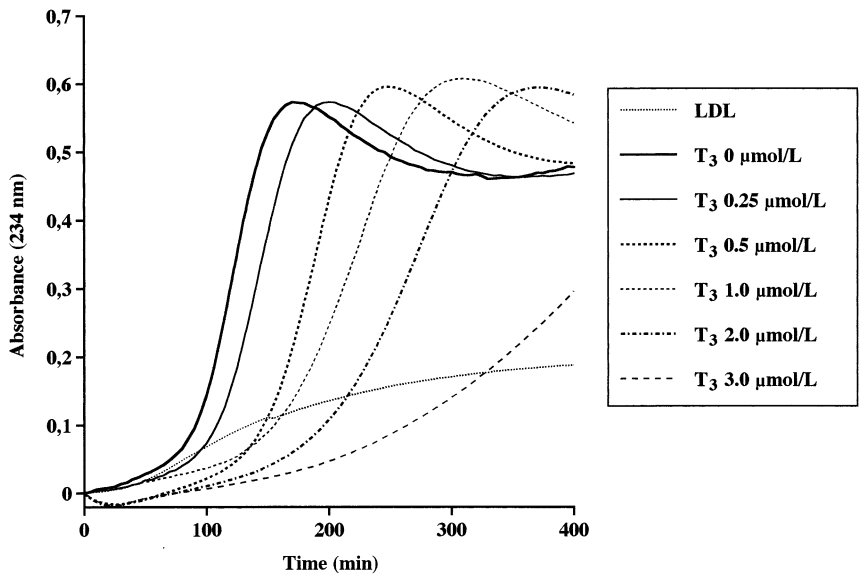


FIG. 2. Conjugated diene absorbance kinetics measured during oxidation of LDL (50 mg protein/L) in the air (LDL, no added Cu^{2+}) or induced by $CuCl_2$ (2.5 μmol/L) in the absence or presence of different T_3 concentrations. Each curve is one example of measures obtained during one LDL oxidation test.

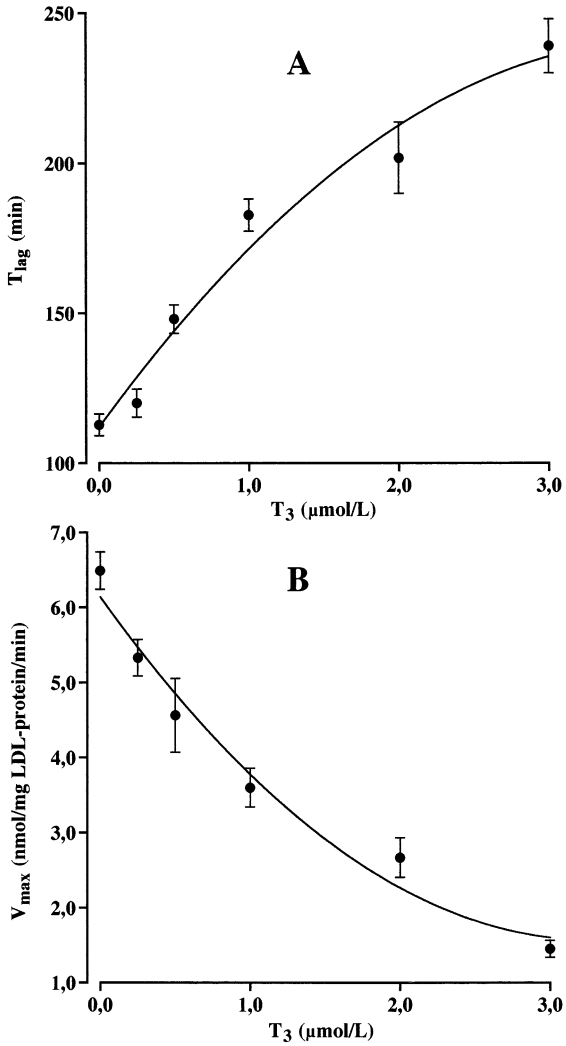


FIG. 3. A) Length of lag phase (T_{lag}) and B) maximum velocity of diene production (V_{max}) during oxidation of LDL (50 mg protein/L) induced by $CuCl_2$ (2.5 μmol/L) in the absence or presence of different T_3 concentrations. Each point is the mean ± SEM of N LDL oxidation tests performed in 3 donors; N = 20, 6, 4, 15, 4 and 3 for 0.0, 0.25, 0.5, 1.0, 2.0 and 3.0 μmol/L of T_3, respectively.

phases, i.e. lag phase, propagation phase and decomposition phase, and maximum absorbance was *ca*. 0.6 u. Abs. In the presence of T_3, the higher the concentrations were, the more the curves tended to the right, indicating that protection against oxidation was concentration-dependent. At 3 μmol/L of T_3, the effect was so considerable that kinetics was not completed for the studied length. Taking into account all the kinetics in the absence or in the presence of the different T_3 concentrations, there were no differences between the D_{max}.

Figure 3 shows relationships between T_{lag} or V_{max} and T_3 concentration obtained during LDL oxidation tests performed in three donors whose oxidation parameters in the absence of T_3 were similar. T_{lag} increased ($r = 0.913$, $N = 52$) and V_{max} decreased ($r = 0.857$, $N = 51$) with T_3 concentrations in a quadratic manner ($P < 0.01$). This means that a curve (second degree equation) more accurately describes the phenomenon than a straight line. In other words, there would be a saturation in antioxidant potency at concentrations greater than 3 μmol/L of T_3. The 1 μmol/L of T_3 concentration increased T_{lag} and decreased V_{max} to *ca*. 50%; this concentration was also used to test the antioxidant efficiency of other compounds.

Examples of diene absorbance kinetics measured during LDL oxidation tests in the presence of the different compounds (1 μmol/L) are given in Fig. 4. Absorbance in the presence of LDL alone always slightly increased to reach values of *ca*. 0.2–0.3 u. Abs., as noted previously. The curves obtained with I_2, MIT and DIT were superimposed on that obtained with Cu^{2+} alone (Fig. 4B). All other curves were shifted to the right of that observed with Cu^{2+} alone, whether in the presence of thyronines (Fig. 4A), of DIMIT or $IpTA_2$ (Fig. 4B), or of acetic derivatives (Fig. 4C). However, curve shifting differed from one compound to another and the curve aspects of DIMIT and $IpTA_2$ were also slightly different from those of other compounds, perhaps indicating different antioxidant properties. The antioxidant efficiency of TA_2 and TA_3 was such that maximum absorbance was not reached at 600 min (Fig. 4C).

The values of T_{lag}, V_{max} and D_{max} during LDL oxidation tests with or without 1 μmol/L of the compounds are presented in Table 2. As expected from kinetics (Fig. 4B), I_2, MIT and DIT did not significantly modify the oxidation parameter values obtained in the presence of Cu^{2+} alone, nor was there any difference in D_{max} between all the compounds and Cu^{2+} alone. On the other hand, the T_{lag} of Cu^{2+} alone was increased ($P < 0.01$) by all the compounds except T_4, whereas the V_{max} was decreased ($P < 0.01$) by all the compounds except $IpTA_2$. It is to be noted that the V_{max} of $IpTA_2$ was 16% lower than the V_{max} of Cu^{2+} alone, but the difference was not significant owing to the great variation in V_{max} values of $IpTA_2$ (SEM = 0.68 nmol/mg of LDL-protein/min) and to Dunnett test severity. On average, the increase in T_{lag} and the decrease in V_{max} by the acetic derivatives were greater than those obtained in the presence of thyronines ($P < 0.01$, ANCOVA). Consequently, further comparisons using the Tukey test were made between thyronines, on the one hand, and between acetic derivatives and T_3, on the other.

Figure 5 shows V_{max} as a function of T_{lag} for the thyronines or the acetic derivatives at 1 μmol/L. Each ellipsoid is the mean ± 95% confidence interval for the mean (i.e. $\pm\ t_{0.05} \times$ SEM) of V_{max} (vertical diameter) and T_{lag} (horizontal diameter). This graphical representation permits a rapid assessment of the antioxidant potency of the compounds, i.e. their capacity to increase T_{lag} and/or decrease V_{max}. Among thyronines, T_0, T_2 and T_3 had the same antioxidant efficiency. rT_3 decreased V_{max} to a similar extent as T_3, but increased T_{lag} ($P < 0.01$) to a lesser extent, whereas T_4 decreased V_{max} less ($P < 0.05$) than did T_3. DIMIT strongly increased T_{lag} compared with other thyronines ($P < 0.01$), whereas it had the same effect on

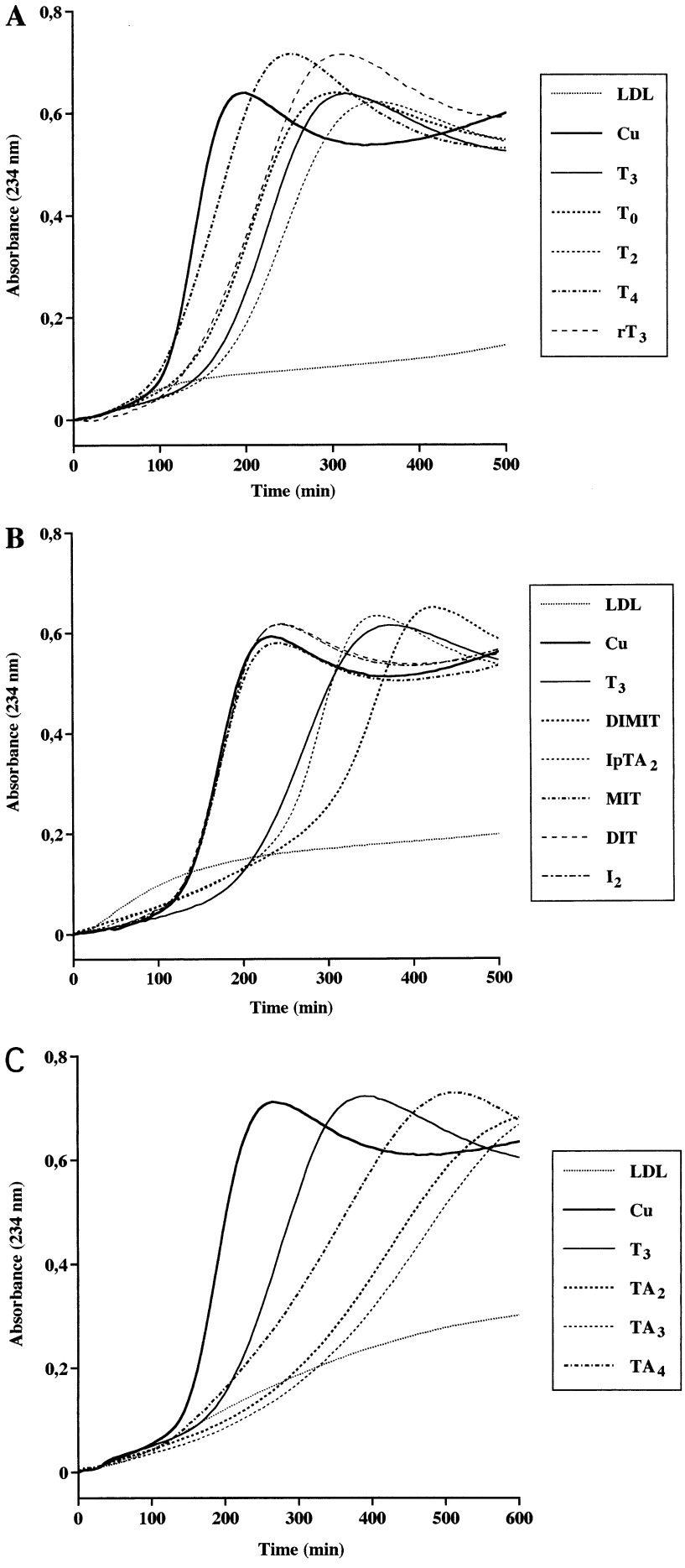


FIG. 4. Conjugated diene absorbance kinetics measured during oxidation of LDL (50 mg of protein/L) in the air (LDL, no added Cu^{2+}) or induced by $CuCl_2$ (2.5 μmol/L) in the absence (Cu) or presence of different thyroid or thyromimetic compounds (1 μmol/L). A, B and C are examples of measures obtained during one LDL oxidation test.

TABLE 2. Length of lag phase (T_{lag}), maximum velocity of diene production (V_{max}) and maximum amount of generated dienes (D_{max}) during oxidation of LDL (50 mg protein/L) induced by $CuCl_2$ (2.5 μmol/L) in the absence (Cu) or in the presence of different thyroid or thyromimetic compounds (1 μmol/L)

	(n)	T_{lag} (min)	V_{max} (nmol/mg LDL-protein/min)	D_{max} (nmol/mg LDL-protein)
Cu	(26)	128 ± 3	6,35 ± 0,23	447 ± 15
I_2	(6)	126 ± 6	6,07 ± 0,70	481 ± 40
MIT	(8)	128 ± 3	5,81 ± 0,27	438 ± 20
DIT	(8)	128 ± 3	5,79 ± 0,30	447 ± 24
T_0	(8)	181 ± 8*	3,64 ± 0,13*	475 ± 19
T_2	(8)	193 ± 7*	3,49 ± 0,17*	479 ± 24
T_3	(23)	192 ± 4*	3,52 ± 0,18*	465 ± 17
rT_3	(8)	155 ± 7*	3,28 ± 0,28*	473 ± 23
T_4	(8)	110 ± 5	4,46 ± 0,25*	531 ± 26
DIMIT	(5)	299 ± 12*	4,14 ± 0,54*	502 ± 43
DIAC	(4)	305 ± 5*	1,25 ± 0,16*	—†
TRIAC	(4)	331 ± 17*	1,16 ± 0,12*	—†
TETRAC	(4)	182 ± 20*	1,40 ± 0,18*	389 ± 53
$IpTA_2$	(5)	273 ± 13*	5,32 ± 0,68	507 ± 45

Means ± SEM of (*N*) LDL oxidation tests (2 tests per subject).
*$P < 0.01$ as compared with Cu (Dunnett test).
†Reaction not completed after 600 min.

V_{max}. Among acetic derivatives, TA_2 and TA_3 were the most active compounds, exhibiting a similar antioxidant efficiency; they increased T_{lag} and decreased V_{max} to a much greater extent than did T_3 ($P < 0.01$). TA_4 had the same effect as TA_3 or TA_2 on V_{max}, whereas its effect on T_{lag} was similar to that obtained in the presence of T_3.

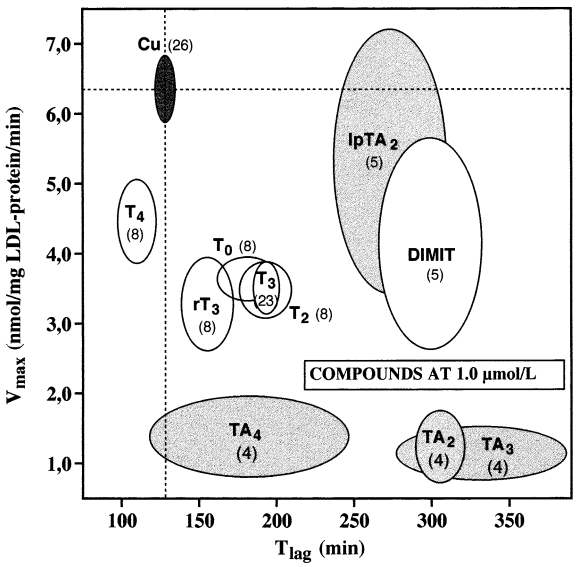


FIG. 5. Maximum velocity of diene production (V_{max}) as a function of the length of the lag phase (T_{lag}) during oxidation of LDL (50 mg of protein/L) induced by $CuCl_2$ (2.5 μmol/L) in the absence (Cu, dark ellipsoid) or presence of 1 μmol/L of different thyronines (white ellipsoids) or acetic derivatives (grey ellipsoids). The center of each ellipsoid is the mean of (N) LDL oxidation tests (2 tests per subject); vertical and horizontal diameters of each ellipsoid are 95% confidence interval for the means of V_{max} and T_{lag}, respectively.

$IpTA_2$ increased T_{lag} more than T_3 did ($P < 0.01$), but less than TA_3 did ($P < 0.01$).

Because diene absorbance kinetics measured in the presence of 1 μmol/L of TA_2 and TA_3 were incomplete (Fig. 4C), LDL oxidation tests were performed in the presence of 0.25 μmol/L of T_3 or physiologic acetic derivatives. At this concentration, there was also no difference in D_{max} between T_3, the derivatives and Cu^{2+} alone. Figure 6 shows V_{max} as a function of T_{lag} for these compounds at 0.25 μmol/L. T_3 increased T_{lag} and decreased V_{max} compared with Cu^{2+} alone ($P < 0.05$). Acetic derivatives had an overall antioxidant efficiency greater than that of T_3 ($P < 0.01$). TA_3 was the most active compound on the two oxidation parameters. TA_2 had the same effect as TA_3 on V_{max}, but increased T_{lag} less than did TA_3 ($P < 0.05$). TA_4 was similar to TA_2 for T_{lag}, but decreased V_{max} less than did TA_2 ($P < 0.05$).

Table 3 presents incubation mixture Cu concentrations before and after filtration and in the presence or out of the presence of 1 μmol/L T_3. Cu concentration in nonfiltered mixtures was slightly less than the theoretical Cu concentration (2.5 μmol/L), but there was no difference between the mixtures with and without T_3. The percentage of total Cu left on the filter can be equated to the percentage of Cu retained in LDL, because LDL were the only substances in the incubation mixtures with molecular weight greater than 30 kDa. T_3 did not significantly modify the percentage of Cu in LDL (47.1% on average), which corresponded to a total LDL-Cu concentration of 22.0 ± 1.3 nmol/mg of LDL-protein ($N = 10$, 3 subjects).

Correcting Cu in LDL alone by Cu in buffer (0.032 ± 0.003 μmol/L; $N = 5$), the initial Cu concentration in LDL, i.e. without addition of $CuCl_2$, can be assessed as

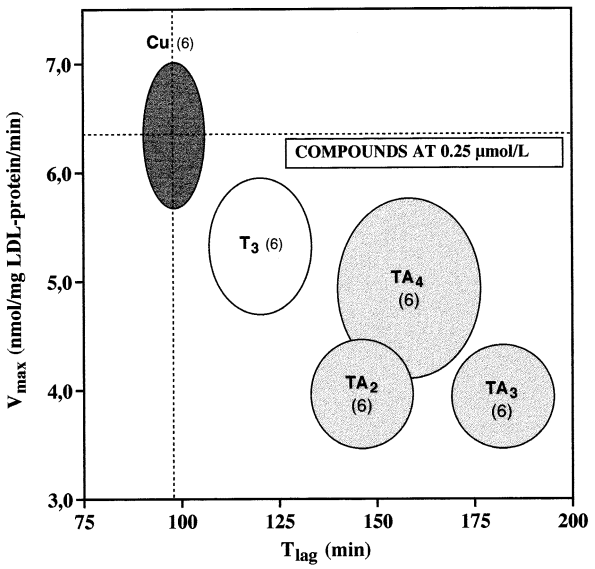


FIG. 6. Maximum velocity of diene production (V_{max}) as a function of the length of the lag phase (T_{lag}) during oxidation of LDL (50 mg of protein/L) induced by $CuCl_2$ (2.5 µmol/L) in the absence (Cu, dark ellipsoid) or presence of 0.25 µmol/L of T_3 (white ellipsoid) or physiologic acetic derivatives (grey ellipsoids). The center of each ellipsoid is the mean of (*n*) LDL oxidation tests (2 tests per subject); vertical and horizontal diameters of each ellipsoid are 95% confidence interval for the means of V_{max} and T_{lag}, respectively.

1.94 ± 0.23 nmol/mg of LDL-protein (N = 3 subjects). Correcting total Cu in LDL in the presence of added $CuCl_2$ by initial Cu in LDL, assuming an apo B-100 molecular mass of 5.5×10^5 and that 80% of the copper was bound in apo B-100 [23], one can calculate that Cu bound by LDL from added $CuCl_2$ amounted to 8.1 ± 0.8 (N = 5) and 9.5 ± 0.8 (N = 5) mol of Cu per mol of apo B-100 for Cu alone and Cu + T_3 experiments, respectively. Cu initially bound in LDL was calculated to be 0.9 ± 0.1 (N = 3) mol Cu per mol apo B-100.

DISCUSSION

Continuous measurement of conjugated dienes allowed us to estimate the effect of thyroid or thyromimetic compounds on different phases of the LDL oxidation process.

TABLE 3. Copper concentrations in filtered (Filtrate Cu) or not (Total Cu) incubation mixture after oxidation of LDL (50 mg of protein/L) for 15 min induced by $CuCl_2$ (2.5 µmol/L) in the absence (LDL+Cu) or in the presence (LDL + Cu + T_3) of 1 µmol/L of T_3

	Total Cu (µmol/L)	Filtrate Cu (µmol/L)	LDL Cu (%)
LDL + Cu	2.39 ± 0.05	1.36 ± 0.08	42.8 ± 3.3
LDL + Cu + T_3	2.28 ± 0.07	1.11 ± 0.10	51.4 ± 3.8

Means ± SEM of 5 LDL oxidation tests in 3 subjects. LDL Cu (%) is the percentage of total Cu left on the filter which stopped substances with a molecular weight or more than 30 kDa, i.e. LDL.

T_{lag} represents the length of the initiation phase during which LDL resist oxidation, V_{max} is the maximum velocity of the propagation phase during which conjugated dienes are intensively produced, and D_{max} is the maximum quantity of generated dienes [7, 21]. The different thyronines had approximately the same effect on oxidation velocity with a V_{max} decrease of *ca.* 45% for T_0, T_2, T_3 and rT_3 and of *ca.* 32% for T_4 and DIMIT, compared with V_{max} of Cu^{2+} alone, whereas they had differing effects on lag phase. In the same way, physiologic acetic derivatives decreased the V_{max} by *ca.* 80%, whereas they had differing effects on T_{lag}. It may be noted that T_{lag} is somewhat related to the slope of the rapid increase of the kinetic profile, i.e. to the oxidation velocity, because it is graphically determined using a tangent to this slope. A decreased oxidation velocity gives a low slope, which tends to produce a low T_{lag}. Thus, the interpretation of T_{lag} should be concomitant to that of V_{max}. This is why we suggest that V_{max} be presented as a function of T_{lag}, using an ellipsoid whose position represents the antioxidant capacity of a compound compared with the ellipsoid obtained in the presence of Cu^{2+} alone (see Figs. 5 and 6). Any ellipsoid under and/or to the right of that of Cu^{2+} may represent a potentially LDL antioxidant compound.

The antioxidant activity was proportional to the hormone concentration in the LDL-containing mixture, because T_{lag} increased and V_{max} decreased as the T_3 concentration increased. However, the relations between oxidation parameters and T_3 concentrations tended towards a plateau, i.e. the lag phase could not be indefinitely increased and diene production could not be completely stopped even by a high T_3 concentration. This indicates that thyroid hormones impair some, but not all, of the mechanisms responsible for peroxidation. Furthermore, we found that D_{max} was similar in LDL oxidized by Cu^{2+} alone or by Cu^{2+} associated with any of the compounds, whatever their concentration. Thus, thyroid hormones and their derivatives slow down, but do not completely stop, fatty acid peroxidation.

Hanna *et al.* [17, 18] reported that T_4, T_3 and rT_3 limited LDL oxidation *in vitro*. They showed that the concentrations of each hormone producing 50% inhibition in the formation of TBARS for 24 hr were similar and equal to 1.10, 1.33 and 1.13 µmol/L for T_4, T_3 and rT_3, respectively. We found that 1 µmol/L of T_3 increased T_{lag} and decreased V_{max} to *ca.* 50%, which confirms that such a T_3 concentration produces an approximately 50% antioxidant effect. However, we showed that T_4 at this concentration did not modify the lag phase and decreased the velocity less than did T_3, whereas rT_3 increased T_{lag} less than did T_3. The differences between our data and those of Hanna *et al.* [17] may be due to the methodology employed. Indeed, TBARS issue from conjugated diene decomposition [8]. Fatty acid peroxidation is completed after 24-hr incubation, as indicated by the time corresponding to the achievement of D_{max} in our LDL oxidation tests, all of which were performed under conditions similar to those of Hanna *et al.*

[17]. Times for the achievement of D_{max} with T_4, T_3 and rT_3 were less than 7 hr, and the maximum time for D_{max} was *ca*. 11 hr with TA_2 and TA_3 at 1 μmol/L. Thus, it is possible that iodothyronines impair conjugated diene decomposition, resulting in low TBARS production. Consistent with this hypothesis is the fact that MDA has been reported to react with the α-amino group of amino acids, particularly tyrosine, to form adducts containing vinylogous amidine linkages [24].

We found that T_3 did not significantly modify the proportion of copper bound to LDL during the initiation phase. One could conclude that iodothyronines do not complex cupric ions, which may be responsible for the LDL-antioxidant effect described for caffeic acid [25]. However, this hypothesis needs further investigation because it was found that iodothyronines in DMSO-water (80:20) solvent at pH 7.4 formed amino acid complexes with Cu^{2+} [26], and that iodothyronines bound to apo B-100 of human LDL [27]. Thus, it is possible that Cu^{2+}-iodothyronine complexes develop into LDL, impairing the prooxidation process. Indeed, this process requires that Cu^{2+} be bound to the protein moiety of LDL, i.e. mainly apo B-100. We calculated that *ca*. 8 mol of Cu added to the incubation mixture were bound by one mol of apo B-100, whereas *ca*. 0.9 mol of Cu per mol of apo B-100 were initially present in dialyzed LDL. These data are very close to those of Kuzuya *et al*. [23] and may correspond to Cu^{2+} pro-oxidant active weak-binding sites and Cu^{2+} nonactive tight-binding sites of apo B-100. However, the greatest care must be taken in interpreting the data on Cu initially present in LDL, because trace amounts of Cu may bind to LDL throughout the LDL isolation procedure.

The 4′-hydroxy diphenyl ether structure may be responsible for the antioxidant activity of thyroid compounds. Indeed, MIT and DIT, which possess a phenolic structure, but no diphenyl ether group, had no significant antioxidant activity. Conversely, all the compounds possessing that group were able to slow down, to varying degrees, LDL oxidation. This may be due to the reversible oxidation of the 4′-hydroxy diphenyl ether structure to a quinoid form, allowing an electron transfer process [28]. In addition, the thyroid derivatives may have free radical scavenging properties, as is known for some phenolic compounds. These properties are due to the capability of these compounds to generate the putative phenoxyl radical, which stability influences the ability of phenols to break a chain of radical reactions. This stability depends on the substituents present on the aromatic ring and in the side-chain [29, 30]. Furthermore, in the present study, different antioxidant activities were observed with different substitution patterns on the 4′-hydroxy diphenyl ether structure.

T_0, T_2 and T_3 acted similarly on the initial phases of oxidation. Thus, the presence of iodine atoms in the 3, 5 or 3′ position does not appear to be essential for antioxidant activity. Moreover, the presence of four iodine atoms decreased the activity, because T_4 showed a moderate antioxidant efficiency. More precisely, the simultaneous presence of substituents in the 3′ and 5′ positions altered antioxidant capacity. Indeed, rT_3 was less active than T_3, and TA_4 had a weaker activity than did TA_2 or TA_3 (see below). Thyroid compounds are known to be highly susceptible to deiodination, especially in the 3′ or 5′ position, thus leading to the release of iodine that may be transformed into oxidant I_2 in the presence of Cu^{2+} and O_2. It might be supposed that I_2 could increase the oxidation of LDL and thus impair the protective action of the 3′ and 5′ di-substituted iodothyronines. However, we found that I_2 had no effect on LDL oxidation mediated by Cu^{2+}. It is to be noted that the presence of a second substituent adjacent to the phenolic hydroxyl (5′ position) reduces thyromimetic activity, perhaps because of an interference with the 4'-hydroxyl hydrogen bond to the thyroid hormone nuclear receptor [28]. Finally, the degree of ionization of the phenolic hydroxyl group may explain the differences observed in antioxidant activity of 3′ and 5′ di-substituted derivatives. Indeed, at pH 7.4, phenolic pKa of T_4, rT_3 and even DIT is *ca*. 6.5, giving a phenolate ion percentage of 80–90%, whereas 3′ mono-substituted derivatives are less than 10% ionized [31].

The results obtained with 0.25 μmol/L of physiologic acetic derivatives were slightly different from those obtained with 1.0 μmol/L, especially for TA_4 (see Figs. 5 and 6). This is not surprising because the increase in T_{lag} and the decrease in V_{max} do not seem to be linearly related to the antioxidant compound concentration, as observed with T_3, and because the incomplete absorbance kinetics obtained in the presence of 1 μmol/L of TA_2 and TA_3 led to difficulties and variability in the measure of parameters, especially T_{lag}. Consequently, it would be preferable to compare acetic derivatives by considering the results at 0.25 μmol/L. However, TA_4 revealed a lower activity than TA_2 or TA_3, confirming that the simultaneous presence of substituents in the 3′ and 5′ positions altered antioxidant capacity. The three physiologic acetic derivatives had a stronger antioxidant capacity than T_3. This was also true, although to a lesser extent, for $IpTA_2$. Thus, antioxidant activity related to the presence of the diphenyl ether structure is strengthened when the amino acid group of the side-chain is replaced by a carboxyl residue. Note here that TA_3 and TA_4 are known to bind more firmly to thyroid hormone nuclear receptor than do T_3 and T_4 [28, 32].

$IpTA_2$ was less active than the other acetic derivatives. This does not seem to be related to the presence of the isopropyl group in the 3′ position because DIMIT, the thyronine that possesses the same substituent in this position, had a greater antioxidant activity than both $IpTA_2$ and T_3. As for the thyromimetic activity of thyroid hormone analogues, it was demonstrated that 3′-substituted derivatives of T_3, e.g. $IpTA_2$, had high affinity for the thyroid hormone receptor when the 3′-substituent was lipophilic and limited in depth to the length of the natural iodo substituent [33]. Moreover, it was shown that 3,5-dimethyl groups may be introduced into the thyronine nucleus without loss of receptor binding, DIMIT being one

of the most active of the halogen-free thyromimetics [28]. This 3,5-dimethyl substitution does not modify the conformation of the molecule necessary to thyromimetic activity, i.e. two mutually perpendicular aromatic rings positioned relative to one another at an angle of *ca.* 120°.

Thus, the LDL-antioxidant potency of the compounds tested here appears to be related to their 3-dimensional molecular shapes and their redox properties. Surprisingly, our observations on structure-antioxidant activity relationships parallel those on structure-thyromimetic activity relationships. This may confirm that electron-transfer capacity associated with a particular conformation is necessary for nuclear receptor binding, which produces thyromimetic effects [28]. However, this may also indicate that the antioxidant efficiency of thyroid derivatives is associated with their binding to LDL, particularly on three sites of apo B-100 [27].

This study demonstrates that thyroid hormones and analogous derivatives can slow down *in vitro* copper-induced LDL oxidation. This antioxidant capacity is related to the 4′-hydroxy diphenyl ether structure, substituted or not by iodine atom. It is increased by the replacement of the amino acid group on the side-chain by a carboxyl group. The physiologic and pharmacologic relevance of these observations merits consideration, because it has been shown that catalytically active cupric ions present in human atheroma lesions are able to stimulate lipid peroxidation [34] and that thyroid hormones bind specifically to apolipoproteins of LDL and other lipoproteins [16, 27].

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