***In vitro* free radical scavenging capacity of thyroid hormones and structural analogues**

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Abstract

It was reported that thyroid hormones decreased Cu^{2+} -induced low-density lipoprotein (LDL) oxidation *in vitro*. Here, we investigated free radical scavenging capacities of thyroid hormones (3,5,3'-tri-iodo-L-thyronine (T_3), thyroxine (T_4) and 3,3',5'-tri-iodo-L-thyronine (rT_3)) and structural analogues (L-thyronine (T_0), 3,5,3'-tri-iodothyroacetic acid (TA_3) and 3,5,3',5'-tetra-iodothyroacetic acid (TA_4)), using three different models of free radical generation. T_0 , T_3 and TA_3 slowed down production of conjugated diene and thiobarbituric acid-reactive substances during LDL oxidation by 2,2'-azobis-[2-amidinopropane] (water-soluble), whereas rT_3 , T_4 and TA_4 had practically no effect. In this system, T_0 was the more active compound. Using a 1,1-diphenyl-2-picrylhydrazyl (lipid-soluble) test, all compounds also

revealed free radical scavenging capacities, but rT_3 , T_4 and TA_4 were more active than T_0 , T_3 and TA_3 . T_3 was able to scavenge superoxide anion and hydroxyl radicals generated in an aqueous phase by a xanthine-xanthine oxidase system, as measured by electron paramagnetic resonance spectroscopy. It may be concluded that: (1) thyroid hormones and analogues with a 4'-hydroxy diphenylether structure have free radical scavenging capacities, (2) this property is influenced by the number of iodines on the phenolic ring, and (3) thyroid hormone scavenging capacity should not be the only mechanism explaining their protective effect on Cu^{2+} -induced LDL oxidation. The physiological significance of the findings is discussed.

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Introduction

It is well-known that thyroid hormones play a role in lipid metabolism. Hypothyroidism is associated with hypercholesterolemia and increased plasma low-density lipoprotein (LDL) concentrations, which are reversed by thyroid hormone treatment (O'Brien *et al.* 1997). In contrast, hyperthyroidism leads to low total and LDL cholesterol concentrations, which are normalized by radioactive iodine treatment (Kung *et al.* 1995). Increased LDL cholesterol is a major risk factor for atherosclerosis and many data support a proatherogenic role for oxidized LDL (Steinberg 1997). Thus the lipid metabolism perturbations observed during hypothyroidism increase atherogenic risk whereas treatment of hypothyroid patients with thyroid hormone reduces it (Martinez Triguero *et al.* 1998). As the oxidizability of LDL was reported to be increased in hypothyroidism (Diekmann *et al.* 1998), it may also contribute to induce the atherogenic process.

The *in vitro* demonstration that thyroid hormones limit the oxidative modifications of LDL was first reported by Hanna *et al.* (1993). They found that thyroxine (T_4), 3,5,3'-tri-iodothyronine (T_3) and 3,3',5'-tri-iodothyronine (rT_3) similarly decreased Cu^{2+} -induced LDL oxidation. Using the same pro-oxidant system, we previously showed that not only thyroid hormones, but also their acetic derivatives and thyronines lacking iodine, had LDL-antioxidant properties (Chomard *et al.* 1998). More precisely, our data suggested that the antioxidant efficiency was related to the 4'-hydroxydiphenylether structure and depended upon the nature and the position of substituents in this structure. However, the mechanism by which these compounds act is at present not elucidated.

Antioxidants that protect lipid LDL from peroxidation may act in different ways, including sparing of endogenous antioxidant vitamins within the LDL particle, metal chelating activity or free radical scavenging. The aim of the present study was to investigate the *in vitro* free radical

scavenging capacities of thyroid hormones and structural analogues: iodotyrosine, precursor of thyroid hormones (monoiodotyrosine (MIT)); thyronines (L-thyronine (T_0 , no iodine), T_3 , rT_3 and T_4); and acetic catabolites of thyroid hormones (3,5,3'-tri-iodothyroacetic acid (TA_3) and 3,5,3',5'-tetra-iodothyroacetic acid (TA_4)). Butylated hydroxytoluene (BHT), a common phenolic antioxidant, was used as antioxidant control.

Experiments were performed using three different models of free radical generation. In one experiment, LDL oxidation was initiated by the water-soluble azoinitiator, 2,2'-azobis-[2-amidinopropane] dihydrochloride (AAPH), which generates alkylperoxyl radicals directly by temperature-dependent unimolecular decomposition within the aqueous phase (Rice-Evans *et al.* 1996). The peroxyl radicals are rapidly decomposed to alkoxyl radicals (Kraiev *et al.* 1996). In another experiment, we used 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable, lipid-soluble free radical that exhibits a strong absorption at 516 nm that is reduced when DPPH reacts with a free radical scavenger (Blois 1958). Finally, using a xanthine oxidase system as a source of superoxide anions and hydroxyl radicals, the scavenging effect of T_3 in aqueous solution was investigated by electron paramagnetic resonance (EPR) spectroscopic detection.

Materials and Methods

Materials

Thyroid hormones and their analogues, BHT, 2-thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetramethoxypropane, DPPH and probucol were purchased from Sigma (La Verpillère, France); AAPH was from Interchim (Montluçon, France). Superoxide dismutase (SOD) (EC 1.15.1.1), mannitol, xanthine and xanthine oxidase (EC 1.1.3.22) were purchased from Sigma. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was also purchased at Sigma, and was purified through double distillation and stored under nitrogen at -70°C before use.

AAPH-induced LDL oxidation experiments

LDL preparation Human plasma was taken into citrate-phosphate-dextran from healthy volunteers selected by the Blood Transfusion Center of Burgundy, France. The plasma was supplemented with 1 g/l EDTA- Na_2 . LDLs (density 1.019–1.063 g/ml) were immediately isolated by density-gradient ultracentrifugation at 4°C , as previously described (Chomard *et al.* 1998). Fresh LDLs were kept under argon and in darkness at 4°C for up to 15 days. Before use, the LDL-containing fraction was dialyzed in the darkness for 24 h at 4°C against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl,

0.01% EDTA and 1% chloramphenicol. The buffer was first made oxygen-free by passage through a Millipore filter, followed by purging with nitrogen for 30 min.

Compound preparations Thyroid hormones and their analogues were dissolved in 300 μl 11 M NH_4OH and 300 μl ethanol, which were quickly made up to 10 ml with extra-pure water. BHT was dissolved first in 300 μl ethanol, then in 300 μl 11 M NH_4OH , and the mixture was made up to 10 ml with ethanol. The 1 mM solutions obtained (pH 9.2) were kept for up to 5 days at 4°C before being further dissolved 1/10 in water as required for the different tests.

LDL oxidation and conjugated diene measurement

The protein concentration of dialyzed LDL was measured by the method of Lowry modified by Markwell *et al.* (1978). Dialyzed LDLs were diluted in 0.02 M phosphate buffer, pH 7.4, without EDTA, in order to obtain 0.5 g LDL-protein per liter. Then 860 μl EDTA-free buffer, 20 μl 75 mM AAPH, 20 μl diluted compound and 100 μl diluted LDL were successively added to quartz cuvettes. A cuvette with AAPH alone was used as a control for the absorbance variation of AAPH, a cuvette with LDL alone was used as a control for LDL particle integrity and buffer purity, and a cuvette with LDL and AAPH was used as oxidation control (no compound). Conjugated diene production kinetics were determined by monitoring the change in absorbance at 234 nm and 37°C with a Uvikon 930 spectrophotometer (Kontron, Milan, Italy) equipped with an automated sampler, allowing for 12 samples to be measured simultaneously. Absorbance was measured against the buffer and recorded every 5 min for 500 min. Final concentrations in the incubation mixture were 50 mg LDL-protein/l, 1.5 mM AAPH and 2 μM studied compounds, except for T_3 , which was tested at different concentrations (0.25–4.00 μM). The final pH of the incubation mixture was 7.4.

The experiments were performed using freshly dialyzed LDL. Each LDL oxidation test systematically consisted of one cuvette with AAPH alone, one cuvette with LDL alone, one cuvette with LDL and AAPH (called 'AAPH'), and additional cuvettes with LDL, AAPH and one of the studied compounds (called 'compound').

The absorbances of each cuvette were corrected by subtracting the corresponding initial absorbance and then the absorbance variation of AAPH alone was also subtracted, except for LDL alone. Corrected data were used to plot the diene absorbance against time and to calculate the changes in absorbance/min as a function of time – that is, the oxidation velocities. Three parameters of LDL oxidation were considered (Gieseg & Esterbauer 1994): the duration of the lag phase (T_{lag} ; determined graphically by the time intercept of the tangents to the slow and rapid increase in 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 $29\,500\text{ mol}^{-1}\text{ liter cm}^{-1}$.

Because LDLs isolated from different donors exhibit different oxidizability characteristics, the data of oxidation parameters in the presence of the compounds were corrected with the data in the absence of the compounds. The corrected parameters were indicated by the symbol Δ (ΔT_{lag} , ΔV_{max} , ...). Consequently, the data of oxidation parameters in the presence of AAPH without compounds were set at 0, and the more the oxidation parameter value of a compound was different from 0, the more this compound had an effect on the parameter concerned.

LDL oxidation and thiobarbituric acid-reactive substances measurement Mixtures similar to those prepared for measurement of conjugated diene were placed in darkness at 37°C . At 1, 2, 4, 6 and 8 h, 500 μl aliquots were taken and placed in Pyrex centrifuge tubes with screw caps. LDL oxidation was stopped by adding 50 μl EDTA-BHT (11 and 2.2 mM respectively) and by storing the closed tubes at 4°C . One milliliter freshly prepared thiobarbituric acid-reactive substances (TBARS) reagent was added, and the closed tubes were mixed and heated at 100°C for 15 min. The TBARS reagent was 100 ml of trichloroacetic, thiobarbituric and chlorhydric acids (13.6:0.36:2.4%, w/v) which were mixed and purged under nitrogen for 30 min. Then the tubes were cooled and centrifuged at 2500 r.p.m. for 15 min, and the absorbance of the supernatant was measured at 532 nm against a control tube without LDL oxidation mixture. Quantification of TBARS was performed by comparison with a standard curve of malondialdehyde equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. This curve was obtained in the same conditions as above, using the EDTA-free buffer in place of the LDL oxidation mixture, and ranged from 0 to 8 μM .

DPPH experiments

The experiments were performed in absolute ethanol. A 200 μM DPPH solution was kept for one night at 4°C until dilution to 50 μM as required. Probucol, a potent DPPH radical scavenger (Hiramatsu *et al.* 1994), was used as a control for 100% scavenging. Probucol, BHT, thyroid compounds and their analogues were dissolved in 100 μl 11 M NH_4OH and quickly made up to 2 ml with absolute ethanol to obtain a 5 mM concentration. To plastic microcuvettes, we successively added 1000 μl DPPH dilution and 10 μl compound solutions or ethanol (DPPH alone). The absorbance of each mixture was simultaneously measured, every 1 min for 30 min at 20°C , at 516 nm against ethanol, using the previously described Uvikon 930 spectrophotometer.

The absorbance kinetics showed that 30 min was the incubation time necessary to reach a stable absorbance for

DPPH alone and for DPPH in the presence of probucol. Therefore, the percentage of radical scavenging against probucol was calculated with absorbances at 30 min as $100 \times (\text{AD} - \text{AC})/(\text{AD} - \text{AP})$, where AD was the absorbance of DPPH alone, and AC and AP the absorbances in the presence of the compounds and probucol respectively.

Hydroxyl and superoxide anion radical scavenging experiments

Free radical generation was characterized by EPR spin trapping techniques using the spin trap DMPO. EPR spectra were recorded in a flat-type quartz cell at 37°C with a Bruker ESP 300-E X-band spectrometer using a TM110 cavity. The following parameters allowed optimal detection of the signals: modulation frequency, 100 kHz; modulation amplitude, 0.8 G; microwave power, 20 mW; time constant, 0.16 s; scan rate, 1.19 G/s. Relative radical concentrations (in arbitrary units, AU) were determined by the measurement of the intensity of the two central lines, on spectra recorded with identical spectrometer settings.

Free radical generation was performed in 50 mM phosphate buffer adjusted to pH 7.4. Hydroxyl radical was generated by a xanthine-xanthine oxidase system (25 μM –2.5 mIU/ml) with addition of Fe/EDTA (5/10 μM) in the presence of the spin trap DMPO (5 mM). The superoxide anion generating system was also xanthine-xanthine oxidase (500 μM –50 mIU/ml), but in the presence of catalase (500 IU/ml) and deferoxamine (1 mM), to avoid the conversion of superoxide anion to hydroxyl radical through spontaneous dismutation and Fenton reaction. The DMPO was used at the concentration of 50 mM. T_3 was first dissolved in NH_4OH , without ethanol, diluted in phosphate buffer, and added to the medium before the xanthine, which starts the enzymatic reaction. Comparisons were made with blanks obtained in identical pH conditions.

Initiation of the spectra acquisition began 75 s (hydroxyl) and 60 s (superoxide) after initiation of the reaction. EPR spectra of the spin-adducts DMPO-OH and DMPO-OOH, corresponding respectively to the adducts of the hydroxyl radical and the superoxide anion radical with DMPO, were recorded. The height of the signal was evaluated and the IC_{50} , corresponding to the substance concentration inducing a signal height reduction of 50%, was determined for each free radical generating system.

Statistics

Results are presented as means $\pm$ S.E.M. The levels of significance chosen were $P < 0.05$ (*) and $P < 0.01$ (**). Statistical analyses were performed by analysis of variance (ANOVA) using the Systat program (Deltasoftware, Meylan, France). The *post-hoc* two-tailed Dunnett test was used to

Figure 1 Duration of lag phase (T_{lag}) (A) and maximum velocity of diene production (V_{max}) (B) during oxidation of LDL (50 mg protein/l) induced by AAPH (1.5 mM) in the presence of different concentrations of T_3 . Each point is the mean $\pm$ S.E.M. of five experiments expressed in variations (Δ) against AAPH without T_3 .

assess significant differences in parameters between each studied compound and corresponding control. The *post-hoc* two-tailed Tukey test was then used to determine if there were any differences among active compounds.

Results

AAPH-induced LDL oxidation experiments

As T_3 concentration increased from 0.25 to 4.0 μ M, ΔT_{lag} increased (Fig. 1A) and ΔV_{max} decreased (Fig. 1B) in an approximately linear manner: T_3 had an antioxidant effect that was proportionate to its concentration. At 2.0 μ M T_3 , ΔT_{lag} was increased by about 50% as compared with AAPH without T_3 . Furthermore, at this T_3 concentration, a slight break appeared in the slope of each relationship. This concentration was then used for the study of all the other compounds.

Examples of diene absorbance kinetics measured during one LDL oxidation experiment in the presence of the

different compounds (2 μ M) are given in Fig. 2. In the absence of AAPH, LDLs were slightly oxidized by air, whereas in the presence of AAPH, absorbance kinetics revealed the three characteristic phases of LDL oxidation: lag phase, propagation phase and decomposition phase. In the presence of AAPH and MIT, the kinetic was slightly shifted to the left of that obtained with AAPH alone, whereas the kinetics obtained in the presence of rT_3 , T_4 and TA_4 were slightly shifted to its right. Note the particular aspect of these three kinetics at the beginning of the reaction, making difficult the measurement of T_{lag} . In the presence of T_0 , T_3 , TA_3 and BHT, the kinetics were clearly shifted to the right, indicating the antioxidant efficiency of these compounds, particularly T_0 . Finally, taking into account all the kinetics except that of LDL alone, maximum absorbance variations, which correspond to the D_{max} parameter, were approximately similar.

Values of the LDL oxidation parameters in the presence of the compounds, as expressed in variations against AAPH without compound, are presented in Table 1. MIT, T_4 and TA_4 did not significantly modify the oxidation parameter values, except for D_{max} , which was slightly increased. In the same way, rT_3 had no effect except on T_{lag} , which was somewhat increased. In contrast, T_0 , T_3 and TA_3 had no significant effect on D_{max} , but clearly increased T_{lag} and decreased V_{max} , whereas BHT was only efficient in increasing T_{lag} . Using the Tukey test to differentiate among active compounds, rT_3 and T_0 increased T_{lag} less ($P < 0.01$) than did T_3 , BHT and TA_3 , whereas T_3 and TA_3 decreased V_{max} less ($P < 0.05$) than did T_0 .

Figure 3 summarizes the global antioxidant potency of the compounds – that is, their capacity to increase T_{lag} , decrease V_{max} , or both. Each ellipsoid is the mean $\pm$ S.E.M. of V_{max} (vertical diameter) and T_{lag} (horizontal diameter), as expressed in variation percentages against AAPH without compound. The point of intersection of 0 values corresponds to AAPH alone; the more the ellipsoids are under or to the right of the AAPH point, the more the corresponding compound has an antioxidant efficiency. This allows clear identification of three groups among the thyronines: rT_3 , T_4 and TA_4 which had very little, if any, effect; T_3 and TA_3 which increased T_{lag} about 90% and decreased V_{max} about 23%; and T_0 which increased T_{lag} 67% and decreased V_{max} 59%. As a comparison, BHT increased T_{lag} as TA_3 did (92%), but had no effect on V_{max} .

Production of TBARS during AAPH-induced LDL oxidation is shown in Fig. 4. When AAPH was omitted, TBARS increased slowly, reaching a value of about 8 nmol/mg LDL protein after 8 h of incubation. In the presence of AAPH, TBARS increased considerably after 1 h, reaching a maximum of about 30 nmol/mg LDL protein at 4 h, then decreasing slowly. In the presence of the compounds, amounts of TBARS at 1 h were similar to those obtained with AAPH alone, whereas they were

Figure 2 Example of conjugated diene absorbance kinetic measured during one oxidation experiment of LDL (50 mg protein/l) in air (LDL, no added AAPH) or induced by AAPH (1.5 mM) in the absence (AAPH) or presence of BHT and different thyroid analogues (2 μ M).

slightly increased after 4 h. However amounts of TBARS were significantly decreased at 2 h with T_0 , T_3 , TA_3 and BHT, and at 4 h with T_0 . Note that TBARS production kinetics were somewhat similar to those obtained during measurements of conjugated diene (Fig. 2), that of MIT

being shifted to the left of that obtained with AAPH alone and those of the others being more or less shifted to its right, reaching their maximum at about 6 h.

Table 1 Duration 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 AAPH (1.5 μ M) in the presence of BHT and different thyroid compounds (2 μ M). Values are means $\pm$ S.E.M. of seven experiments

	T_{lag} (min)	V_{max} (nmol/mg LDL-protein per min)	D_{max} (nmol/mg LDL-protein)
AAPH	63 ± 4.0	6.3 ± 0.8	379 ± 30
ΔMIT	-8 ± 2	$+0.2 \pm 0.2$	$+42 \pm 10^*$
ΔT_0	$+38 \pm 7^{**}$	$-3.6 \pm 0.4^{**}$	$+16 \pm 12$
ΔT_3	$+51 \pm 5^{**}$	$-1.5 \pm 0.5^*$	$+19 \pm 9$
ΔrT_3	$+22 \pm 4^*$	-0.6 ± 0.4	$+25 \pm 10$
ΔT_4	$+11 \pm 5$	-0.7 ± 0.6	$+35 \pm 9^*$
ΔTA_3	$+55 \pm 5^{**}$	$-1.8 \pm 0.6^*$	$+30 \pm 13$
ΔTA_4	$+15 \pm 3$	-1.2 ± 0.3	$+51 \pm 10^{**}$
ΔBHT	$+52 \pm 9^{**}$	$+0.2 \pm 0.3$	$+15 \pm 9$

Δ , Variations against AAPH without compound (AAPH). * $P < 0.05$, ** $P < 0.01$ compared with AAPH (Dunnett test).

Figure 3 Maximum velocity of diene production (V_{max}) as a function of the duration of the lag phase (T_{lag}) during oxidation of LDL (50 mg protein/l) induced by AAPH (1.5 mM) in the presence of 2 μ M MIT (dark ellipsoid), different thyronines (white ellipsoids), acetic derivatives (deep-gray ellipsoids) and BHT (light-gray ellipsoid). The center of each ellipsoid is the mean of seven experiments, expressed in variation (Δ in %) against AAPH without compound; vertical and horizontal diameters of each ellipsoid are S.E.M. of ΔV_{max} and ΔT_{lag} respectively.

Figure 4 Production of TBARS during oxidation of LDL (50 mg protein/l) in air (LDL, no added AAPH) or induced by AAPH (1.5 mM) in the absence (AAPH) or presence of BHT and different thyroid analogues (2 µM). * $P < 0.05$ and ** $P < 0.01$ compared with AAPH without thyroid analogue at the same time (Dunnett test).

DPPH experiments

All the compounds, except MIT, revealed DPPH radical scavenging capacities (Fig. 5). However, radical scavenging activity of the thyroid compounds was lower than that of BHT (34%). Among them, T_0 , T_3 and TA_3 had the weakest activity (about 8%); T_4 (14%) had greater activity than T_0 and T_3 ($P < 0.05$; Tukey), and rT_3 and TA_4 had the most marked activity (about 21%), which differed from that of T_0 , T_3 and TA_3 ($P < 0.01$; Tukey).

Hydroxyl and superoxide anion radical scavenging experiments

The spectra corresponding to DMPO-OOH and DMPO-OH are shown in Fig. 6. The IC_{50} values for the superoxide (DMPO-OOH) and hydroxyl (DMPO-OH) radical adducts are indicated in Fig. 7. Figures 6A and 7A show the diminution in the peak height of the DMPO-OOH signal for T_3 over a range of concentrations. T_3 was effective as a superoxide scavenger at a concentration less than 2.5 mM, but no total inhibition of the signal was seen at greater concentrations, because of the insolubility of the compound. The IC_{50} of T_3 for superoxide anion was found to be 2.2 mM; as a comparison, the water-soluble analogue of vitamin E, Trolox, showed an IC_{50} of 1 mM (Walker *et al.* 1998). T_3 also scavenged hydroxyl radicals at concentrations less than 0.4 mM, as seen in Figs 6B and 7B. The IC_{50} of T_3 was

around 0.22 mM. As a comparison, the IC_{50} of mannitol for scavenging the hydroxyl radical is 8 mM.

Figure 5 Percentage of radical scavenging against probucol (100%) of BHT and different thyroid analogues. Radical scavenging was obtained by measuring the decrease in absorbance of DPPH (50 µM) after 30 min in the presence of the compounds (50 µM). Each value is the mean $\pm$ S.E.M. of 11 experiments. All the values are different from 0 ($P < 0.01$), except that of MIT. * $P < 0.05$ and ** $P < 0.01$ compared with BHT for active thyroid analogues (Dunnett test).

Figure 6 EPR spectra of the radical adduct of superoxide (A) and hydroxyl (B) with DMPO. (A) EPR signal corresponding to DMPO-OOH ($a_N=14.3$ G, $a_H\beta=11.3$ G, $a_H\gamma=1.4$ G, $g=2.006$) obtained with xanthine (500 μ M), xanthine oxidase (50 mIU/ml), catalase (500 IU/ml), deferoxamine (1 mM) and DMPO (50 mM). 1=blank; 2=plus T_3 (2.5 mM); 3=same as 1, but without xanthine. (B) EPR signal corresponding to DMPO-OH ($a_N=a_H=14.9$ G, $g=2.006$) obtained with xanthine (25 μ M), xanthine oxidase (2.5 mIU/ml), Fe/EDTA (5/10 μ M) and DMPO (5 mM). 1=blank; 2=plus T_3 (200 μ M); 3=same as 1, but without xanthine.

Discussion

It has been reported that thyroid hormones and structural analogues limited *in vitro* LDL oxidation induced by Cu^{2+} (Hanna *et al.* 1993, Chomard *et al.* 1998) or human endothelial cells (Hanna *et al.* 1995). Here, we show that it was also true using the free radical generator, AAPH, and that thyroid compounds revealed free radical scavenging properties.

During Cu^{2+} -induced LDL oxidation, binding of Cu^{2+} to apolipoprotein B100 of LDL is necessary for the generation of the initiating radical (Kuzuya *et al.* 1992, Gieseg & Esterbauer 1994, Wagner & Heinecke 1997). Thus antioxidants that inhibited Cu^{2+} -induced LDL oxidation may first act by chelating copper, as reported for caffeic acid (Nardini *et al.* 1995) or flavonoids (Brown *et al.* 1998). In the present study, thyroid compounds decreased the production of conjugated diene and TBARS during LDL oxidation induced by AAPH. This clearly indicates that these compounds exert an antioxidant effect on LDL oxidation independently of metal-ion chelation. However, it may be suspected that thyroid compounds also disturb Cu^{2+} binding on apolipoprotein B100. Indeed, during Cu^{2+} -induced LDL oxidation (Chomard *et al.* 1998), i) TA_3 had a much greater antioxidant effect than T_3 , whereas it was similar in the present study, ii) TA_4 strongly decreased V_{max} by about 80%, more than T_3 (45%), whereas it only decreased it by 9% here, and iii) rT_3 had an antioxidant effect similar to that of T_3 , whereas it had practically no effect here.

Among the oxidation parameters, T_{lag} represents the lag-phase during which endogenous lipophilic antioxidants such as α -tocopherol prevent radical chain propagation, whereas V_{max} is the maximum velocity of the rapid propagation phase occurring when endogenous antioxidants are consumed. As T_3 , TA_3 and T_0 increased T_{lag} , one may think that they act by sparing endogenous lipophilic antioxidants. Although possible, this mechanism

does not account for the entire antioxidant effect of the thyroid compounds. Firstly, they also decreased V_{max} , whereas BHT (an α -tocopherol-sparing compound) did not. Indeed, it has been reported that BHT at 2 μ M, the same concentration as in the present study, decreased the rate of α -tocopherol degradation during AAPH-induced LDL oxidation (Bowry & Stocker 1993). Secondly, the kinetic profile for the initiation phase in the presence of BHT was clearly horizontal until the propagation phase (Fig. 2), whereas those with T_3 , TA_3 and T_0 rapidly curved, suggesting that the radical chain-breaking capacity of endogenous antioxidants was not highly preserved.

Thus T_3 , TA_3 and T_0 should essentially act as free radical scavengers in AAPH-induced LDL oxidation. AAPH resides almost entirely in the aqueous phase and generates, at a constant rate, a continuous flux of free radical ($AAPH^\bullet$) which rapidly reacts with dioxygen to give the initiating alkylperoxyl radical ($AAPH-OO^\bullet$) (Rice-Evans *et al.* 1996, Thomas *et al.* 1997). This alkylperoxyl radical must then diffuse into the LDL particle to initiate peroxidation. As active thyroid compounds were able to slow down the initiation phase, and owing to the assumption that they did not essentially spare endogenous antioxidants, one may think that they scavenged the AAPH-derived alkylperoxyl radical directly in the aqueous phase. However, because they particularly decreased V_{max} of the propagation phase, they would also be able to scavenge peroxyl radicals within the lipid phase. This is compatible with the relatively amphiphilic character of thyroid compounds in our conditions of low molar concentrations at pH 7.4 and 37 °C.

Our data obtained in EPR experiments confirmed that T_3 was able to scavenge free radicals generated in an aqueous buffer. It was able to scavenge superoxide radical at a concentration range similar to that described for Trolox, the water-soluble analogue of vitamin E. However, the IC_{50} of T_3 for scavenging $O_2^{\bullet-}$ (2.2 mM) was

Figure 7 Inhibition of spin adducts signal height by increasing T₃ concentrations. (A) Inhibition of superoxide anion adducts (DMPO-OOH). (B) Inhibition of hydroxyl radical adducts (DMPO-OH). Each point represents the mean of four individual measurements.

almost twice that of Trolox. The potency of the hormone for scavenging the very active hydroxyl radical was high. In fact, the IC₅₀ was 0.22 mM, which was a concentration 36-fold lower than that observed for mannitol. As a comparison, in a different buffer, the IC₅₀ of Trolox for scavenging $\bullet$ OH was about 1 mM (Walker *et al.* 1998). These results suggest that T₃ may be a very potent scavenger against the oxygen-derived free radicals occurring during physiopathological processes.

In contrast, during the DPPH experiments, T₃ revealed relatively weak scavenging capacity for the low-reactive DPPH radical, with regard to BHT and probucol. Furthermore and surprisingly, T₄, TA₄ and rT₃ revealed DPPH radical scavenging capacities greater than those of T₀, T₃ and TA₃, whereas these last compounds had the greatest antioxidant effect on the AAPH-induced LDL oxidation. It is obvious that the number of iodines on the phenolic ring (in 3' and 5') plays a role in free radical scavenging properties of the thyroid compounds. T₄, TA₄

and rT₃, which are 3',5'-di-iodosubstituted, had similar scavenging properties in either DPPH or AAPH experiments; this was also true for T₃ and TA₃, which are 3'-mono-iodosubstituted. The antioxidant behavior of a compound depends on its different functional groups which affect its lipophilicity and ionization (Macfaul *et al.* 1996), and consequently it also depends on the reaction solvent. The DPPH experiments were performed in absolute ethanol, whereas the AAPH experiments were performed in aqueous buffer at pH 7.4. In this latter medium, T₄ and rT₃ exist at about 80% under the phenolate form, whereas T₃ and T₀ dissociate only 8% and 0.7% respectively (Jorgensen 1978). Thus, during the AAPH experiments, 3',5'-di-iodosubstituted compounds may primarily be present in the aqueous medium and may not be able to scavenge peroxy radicals within the lipid phase. Furthermore, the dissociation into the phenolate form might be responsible for an inefficiency of free radical scavenging.

The 4'-hydroxydiphenylether structure of thyroid compounds was necessary for the free radical scavenging activity. MIT, which possesses the lateral chain and the mono-iodosubstituted phenolic ring of T₃, had no effect either on the production of conjugated diene and TBARS during the AAPH-induced LDL oxidation, or on the DPPH free radical. Conversely T₀, which possesses the basic structure of thyroid hormones but without iodine, scavenged free radicals in the two systems.

We found that the maximum amounts of conjugated diene (D_{max}) and TBARS after 4 h incubation were similar, or even increased, in LDLs oxidized by AAPH associated with any of the compounds, as compared with AAPH alone. Thus thyroid compounds slowed down, but did not completely stop LDL oxidation in our experimental conditions. This is not surprising owing to the large AAPH concentration used, which produced 380 nmol/mg LDL-protein of conjugated diene in about 2 h, whereas the compound concentration was only 40 nmol/mg LDL-protein. The most likely mechanism for free radical scavenging of thyroid compounds is the formation of an unstable phenoxyl radical that decomposes to stable hydroquinone and (iodo)tyrosine. This oxidative decomposition, known as ether-link cleavage of iodothyronines, may be a minor pathway of catabolism *in vivo* (Green 1994). Thus a fixed thyroid compound concentration may only scavenge an equal free radical concentration and cannot stop the lipid auto-peroxidation process.

In order to increase measurement sensitivity, AAPH-induced LDL oxidation was performed using a large concentration of pro-oxidant agent on a weak LDL concentration that generated large amounts of conjugated diene and TBARS. As a comparison, LDL TBARS content in normal individuals is about 0.2–0.8 mol/mol LDL (Esterbauer *et al.* 1992, Kontush *et al.* 1996), whereas it was about 20 mol/mol LDL after 4 h of incubation in the present study. Consequently, thyroid compound

concentration was set to 2 μM , an IC_{50} corresponding approximately to the T_3 concentration that increased T_{lag} by 50% or decreased V_{max} by 50% (Fig. 3). Thus the thyroid compound concentration used in the AAPH test cannot be compared with physiological serum concentrations of thyroid hormones, which are 50–150 nM for T_4 , about 2 nM for T_3 , 0.3 nM for rT_3 , and 0.1 nM for TA_3 . However, there is no reason to suppose that such a radical scavenging process does not occur *in vivo*, where LDL oxidation is limited as compared with our experimental conditions. For example, applying conditions near the physiological situation, Hanna *et al.* (1993) reported finding a T_4 IC_{50} of only 0.21 μM on air-oxidized LDL. The small fraction of circulating thyroid hormones that is bound to lipoproteins (Benvenaga & Robbins 1993) may well protect the LDL surface from radical attacks, particularly in the arterial intima, where LDL oxidation primarily occurs (Smith *et al.* 1992, Heinecke 1998, Seiss *et al.* 1999). In hypothyroidism, the decrease in serum thyroid hormones and in T_3 - and T_4 -LDL binding (Benvenaga & Robbins 1996) might contribute to enhanced LDL peroxidation and thus the atherosclerotic process. Indeed, LDL oxidizability has been reported to be increased in hypothyroid patients (Sundaram *et al.* 1997, Costantini *et al.* 1998, Diekmann *et al.* 1998). However, in such patients, modifications of the LDL fatty acid composition, particularly the lower oleic to linoleic acid ratio (Kleinvelde *et al.* 1993), and increases in circulating total and LDL cholesterol (Martinez Triguero *et al.* 1998) may also be factors that favor LDL oxidizability.

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