

# Inhibition of *in vitro* macrophage-induced low density lipoprotein oxidation by thyroid compounds

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## Abstract

Oxidized low density lipoproteins (LDL) are highly suspected of initiating the atherosclerosis process. Thyroid hormones and structural analogues have been reported to protect LDL from lipid peroxidation induced by  $\text{Cu}^{2+}$  or the free radical generator 2,2'-azobis-[2-amidinopropane] dihydrochloride *in vitro*. We have examined the effects of thyroid compounds on macrophage-induced LDL oxidation. Human monocyte-derived macrophages (differentiated U937 cells) were incubated for 24 h with LDL and different concentrations (0–20  $\mu\text{M}$ ) of 3,5,3'-triiodo-L-thyronine ( $\text{T}_3$ ), 3,5,3',5'-tetraiodo-L-thyronine ( $\text{T}_4$ ), 3,3',5'-tri-iodo-L-thyronine ( $\text{rT}_3$ ), the  $\text{T}_3$  acetic derivative (3,5,3'-tri-iodothyroacetic acid;  $\text{TA}_3$ ) or L-thyronine ( $\text{T}_0$ ) (experiment 1). Cells were also preincubated for 24 h with 1 or 10  $\mu\text{M}$  of the compounds, washed twice, then incubated again for 24 h with LDL (experiment 2). Oxidation was evaluated by measurement of thiobarbituric acid-reactive substances (TBARS) and cell viability by lactate dehydrogenase release. In experiment

1,  $\text{T}_0$  had no effect, whereas the other compounds decreased LDL TBARS production, but  $\text{T}_3$  and  $\text{TA}_3$  were less active than  $\text{T}_4$  and  $\text{rT}_3$  ( $\text{IC}_{50}$ :  $11.0 \pm 2.6$  and  $8.1 \pm 0.8$  vs  $1.4 \pm 0.5$  and  $0.9 \pm 0.3$   $\mu\text{M}$  respectively). In experiment 2, the compounds at 1  $\mu\text{M}$  had no effect; at 10  $\mu\text{M}$ ,  $\text{T}_3$  and  $\text{rT}_3$  slightly reduced LDL TBARS production, whereas  $\text{TA}_3$  and  $\text{T}_4$  inhibited it by about 50% and 70% respectively. TBARS released by the cells were also highly decreased by  $\text{T}_3$ ,  $\text{T}_4$ ,  $\text{rT}_3$  and  $\text{TA}_3$  in experiment 1, but only by  $\text{T}_3$  (30%) and  $\text{T}_4$  (70%) in experiment 2. Cell viability was not affected by the compounds except slightly by  $\text{TA}_3$  at 10  $\mu\text{M}$ . The data suggested that the physico-chemical antioxidant capacity of thyroid compounds was modulated by their action on the intracellular redox systems of macrophage. Overall cellular effects of  $\text{T}_3$  led to a reduction of its antioxidant capacity whereas those of  $\text{T}_4$  increased it. Thus  $\text{T}_4$  might protect LDL against cellular oxidation *in vivo* more than  $\text{T}_3$ .

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## Introduction

Many data support the idea that oxidative modifications of low density lipoproteins (LDL) contribute to enhance their atherogenicity (Chisolm & Steinberg 2000, Witztum & Steinberg 2001). Oxidized LDL can attract monocytes that adhere to the intravascular endothelium and migrate to the subendothelial space where they differentiate into macrophages. Scavenger receptors of macrophages, that are not downregulated by cellular cholesterol content, capture oxidized LDL whose lipids accumulate into the cells. Thus cholesterol-loaded macrophages change into foam cells that invade the arterial wall inducing the fatty streak, an early step in atheromatous plaque formation (Kaplan & Aviram 1999).

Among factors responsible for LDL oxidation *in vivo*, monocyte-derived macrophages themselves are likely candidates, particularly at the beginning of vascular lesions. Indeed, they are prominent in arterial lesions and are able to generate reactive oxygen species (ROS) after activation of enzymes such as 15-lipoxygenase, myeloperoxidase or NADPH oxidase (Chisolm *et al.* 1999, Kaplan & Aviram 1999). Transition metal ions such as copper and iron, which are present in atherosclerotic plaques, may also contribute to LDL oxidation (Lamb *et al.* 1995). For example, ferrous ions ( $\text{Fe}^{2+}$ ) induce lipid peroxidation of cultured macrophages, increasing their ability to oxidatively modify LDL (Fuhrman *et al.* 1994).

Hypothyroidism is associated with cardiovascular diseases due to increased plasma total and LDL cholesterol

contents which are normalized by thyroid hormone treatment (O'Brien *et al.* 1997, Martinez Triguero *et al.* 1998). This is also true in patients with mild thyroid failure, i.e. in subclinical hypothyroidism (Danese *et al.* 2000). Oxidizability of LDL was reported to be increased in hypothyroid patients (Sundaram *et al.* 1997, Costantini *et al.* 1998), increasing the atherogenic risk, but normalized by a 3,5,3',5'-tetraiodo-L-thyronine ( $T_4$ ) treatment (Diekman *et al.* 1998). Thus thyroid hormones, which are suspected to increase oxidative stress during hyperthyroidism (Bianchi *et al.* 1999), may also have antiatherogenic properties when they are used in the treatment of hypothyroid states.

Thyroid hormones and structural analogues have been shown to decrease the *in vitro* LDL oxidation induced by cuprous ions ( $Cu^{2+}$ ) (Hanna *et al.* 1993, Chomard *et al.* 1998), the free radical generator 2,2'-azobis-[2-amidinopropane] dihydrochloride (AAPH) (Oziol *et al.* 2001) or human endothelial cells (Hanna *et al.* 1995). However, their effect on macrophage-induced LDL oxidation is unknown. In the present study, we measured LDL oxidation induced by cultured human monocyte-derived macrophages preincubated or not with different thyroid compounds:  $T_4$  and 3,5,3'-tri-iodo-L-thyronine ( $T_3$ ), the thyromimetic-active thyroid hormones; 3,3',5'-tri-iodo-L-thyronine (reverse  $T_3$ ,  $rT_3$ ), the thyromimetic-inactive hormone; 3,5,3'-tri-iodothyroacetic acid ( $TA_3$ ), the acetic catabolite of  $T_3$ ; and L-thyronine ( $T_0$ ), the hormone structure-like compound without iodine. Macrophage oxidation and viability were also appreciated.

## Materials and Methods

### Materials

Thyroid hormones and their analogues, 2-thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetramethoxypropane, phorbol 12-myristate 13-acetate (PMA) and  $FeSO_4$  were purchased from Sigma (Saint-Quentin, France). RPMI-1640 medium, phosphate-buffered saline (PBS), antibiotic and antimycotic solution and fetal calf serum were provided by Life Technologies (Cergy Pontoise, France), whereas Ham's F-10 medium was from Polylabo (Strasbourg, France).

### Macrophage-induced LDL oxidation experiments

**LDL preparation** Human plasma was withdrawn in the presence of citrate-phosphate-dextran from healthy volunteers selected by the Blood French Establishment of Burgundy, France. The plasma was supplemented with 1 g/l EDTA- $Na_2$  and LDL (density=1.019–1.063) were immediately isolated by density-gradient ultracentrifugation for 15 h at 375 000 g and 15 °C (Kleinvelde *et al.* 1992) using a Centrikon T-1190 centrifuge (Kontron

Instruments, Milan, Italy). The purity of LDL was evaluated by 0.5% agarose gel electrophoresis (Paragon Lipogel kit; Beckman, Fullerton, CA, USA). Fresh LDL were kept under argon and in darkness at 4 °C for up to 15 days. Before use, LDL-containing fractions were dialyzed in darkness for 24 h at 4 °C against 0.01 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 (0.45 µm; Saint-Quentin, France) followed by purging with nitrogen for 30 min. The protein concentration of dialyzed LDL was measured by the Lowry method modified by Markwell *et al.* (1978).

**Compound preparations** Thyroid hormones and their analogues were dissolved in 50 µl 0.5 M NaOH which were quickly completed to 10 ml with extra-pure water. The 1 mM solutions obtained were kept for up to 5 days at 4 °C before their further extemporaneous dissolution in extra-pure water. The working solutions obtained ranged from 25 to 1000 µM.

### Monocyte culture and monocyte differentiation into macrophage

Monocytic U937 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The cells were fed every 3 days and cultivated at 37 °C in a humidified atmosphere of 5%  $CO_2$  and 95% air. To ensure exponential growth, cells were resuspended at a density of  $0.5 \times 10^6$  cells/ml in fresh complete medium 24 h before differentiation. Macrophagic differentiation and activation were obtained in six-well culture plates (2.5 ml/well) following 48 h incubation of  $0.45 \times 10^6$  cells/ml with 100 nM PMA in complete medium. The cells were differentiated into 80–90% adherent monocyte-derived macrophages, hereafter denoted as macrophages.

### Oxidation of LDL by macrophages incubated with thyroid compounds

The non-adherent undifferentiated cells, traces of RPMI-1640 medium or fetal calf serum were removed from wells by two 5-min PBS washes. The following were successively added to the wells: 20 µl thyroid compound working solution, 20 µl 250 µM  $FeSO_4$  and 10–20 µl dialyzed LDL in order to obtain a final concentration of 100 µg protein/ml, and total volume was completed to 1 ml with serum-free Ham's F-10 medium without phenol red. The cells were then incubated for 24 h at 37 °C under 5%  $CO_2$  as usual.

No phenol red was added in order to avoid optical interferences with the pink chromophore measured at the time of assay of thiobarbituric acid-reactive substances (TBARS, see below). Note that Ham's F-10 medium contained 3 µM  $Fe^{2+}$  leading to an  $Fe^{2+}$  final concentration of 8 µM. The small volume of added ferrous ions

did not alter the pH of the cell medium. Thyroid compound final concentrations ranged from 0.5 to 20  $\mu\text{M}$ , corresponding to 0.0025 to 0.1% of diluent NaOH.

Several controls were made simultaneously under the same conditions, removing one of the medium components but maintaining the volume of its diluent. Controls without LDL were performed in order to estimate cellular membrane peroxidation. Three wells of each six-well culture plate were used for these controls, whereas the other three wells were used for one tested thyroid compound, i.e. each assay was performed in triplicate. Controls without thyroid compound were made to appreciate a possible effect of the different NaOH concentrations on the cells. Finally, controls without cells allowed the estimation of spontaneous LDL oxidation.

#### **Oxidation of LDL by macrophages preincubated with thyroid compounds**

After the differentiation and the PBS washes described above, cells were preincubated for 24 h as usual in phenol red-free Ham's F-10 in the presence of two concentrations of thyroid compounds (1 or 10  $\mu\text{M}$ ). Then, after two PBS washes, cells were incubated again for 24 h with  $\text{FeSO}_4$  and LDL at the same concentration as previously. Macrophages in these conditions are henceforward denoted as preincubated cells.

In order to control the first incubation, cells were simultaneously incubated for 24 h in phenol red-free Ham's F-10 alone, and then 24 h again with  $\text{FeSO}_4$ , LDL and a thyroid compound. In each case, controls similar to the ones above were performed, except for the controls without cells since spontaneous LDL oxidation was found to be negligible (Fig. 1). Macrophages in these conditions are henceforward denoted as non-preincubated cells.

#### **Measurement of lipid peroxidation**

Lipid peroxidation was estimated by TBARS measurement, using freshly prepared TBARS reagent made of trichloroacetic/thiobarbituric/chlorhydric acids (13.6/0.36/2.4%, w/v). At the end of each experiment, cell supernatants were removed and lipid peroxidation was stopped by the addition of 50  $\mu\text{l}$  EDTA (1 mM final) and 50  $\mu\text{l}$  butylhydroxy-toluene (0.2 mM final) at 4 °C. The mixture was then centrifuged 5 min at 207 *g* to remove any detached cells. The supernatant (550  $\mu\text{l}$ ) and the TBARS reagent (1 ml) were placed in Pyrex centrifuge tubes with a screw cap, and the closed tubes were mixed and heated at 100 °C for 15 min. The tubes were then cooled and centrifuged at 1300 *g* for 15 min, and the absorbance of the supernatant was measured at 532 nm. Quantification of TBARS was performed by comparison with a standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. This curve was obtained in the same conditions as above using the phenol red-free Ham's F-10 in place of the oxidation mixture, and ranged from 0 to 8  $\mu\text{M}$ .

#### *Cell viability test*

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity released by dead cells after cell incubation for 24 h with  $\text{FeSO}_4$ , with or without LDL and with or without thyroid compounds. LDH activity was quantitated over time by spectrophotometric measurement of the production of NADH using a commercially available kit (LDH/LD, ref. 228–10; Sigma). At the end of each experiment, the culture medium was harvested and the cells were scraped and sonicated into PBS (200  $\mu\text{l}$ /well). Extra- and intracellular LDH activities were measured in the culture medium and in the sonicated cell suspension respectively. Cell protein content was determined by the Lowry/Markwell procedure in the cell suspension.

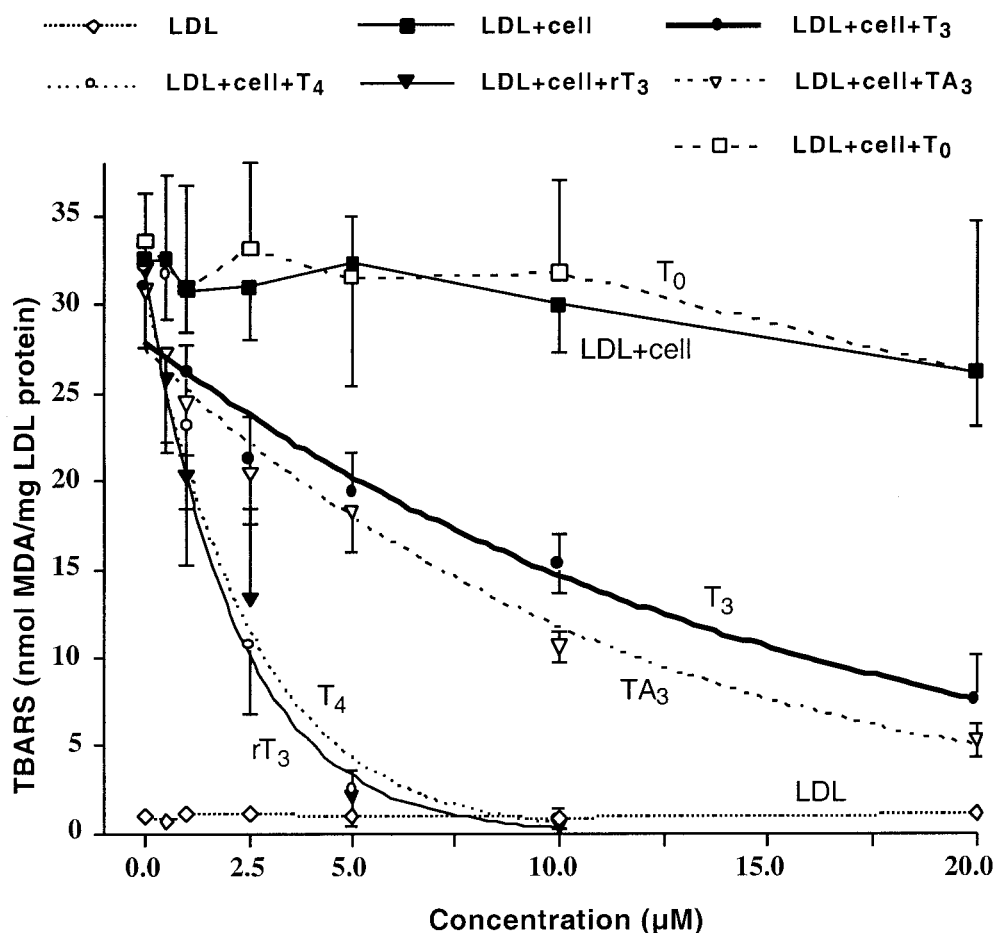
Specific LDH activity (IU/g) was assessed by the ratio between LDH activity (IU/l) and medium protein concentration (g/l). Total specific LDH activity was obtained by adding intra- and extracellular specific activities. Relative intracellular LDH activity was obtained by the ratio between intracellular specific LDH activity and total specific LDH activity. The percentage of viability for one well was then represented by the ratio between its relative intracellular LDH activity and the relative LDH activity of cells in Ham's F-10 alone, considered as the 100% viability.

#### *Statistics*

Results are presented as means  $\pm$  S.E.M. The levels of significance chosen were  $P < 0.05$  and  $P < 0.01$ . Analysis of variance (ANOVA) was performed using the Systat program (Deltasoftware, Meylan, France). Comparisons between variation percentages obtained in the presence of compounds and the 100% theoretical value of controls were done using the two-tailed Student's *t*-test.

#### **Results**

The effect of different concentrations of thyroid compounds on macrophage-induced LDL oxidation for 24 h is shown in Fig. 1. In the absence of cells, LDL peroxidation was negligible, whereas LDL produced about 30–35 nmol TBARS/mg protein in the presence of cells and 0.0025–0.05% of NaOH (thyroid compound diluent). At 0.1% of NaOH corresponding to the 20  $\mu\text{M}$  concentration of thyroid compound, only 26 nmol TBARS/mg LDL protein were produced, indicating that cell pro-oxidant activity was disturbed. This 20  $\mu\text{M}$  thyroid compound concentration was then omitted from further experiments.  $\text{T}_0$  had no effect on macrophage-induced LDL oxidation, whereas all the other compounds decreased it in a concentration-dependent manner. However, LDL TBARS production was strongly and similarly decreased by  $\text{T}_4$  and  $\text{rT}_3$ , becoming negligible between 5 and 10  $\mu\text{M}$



**Figure 1** TBARS production by LDL alone (LDL), LDL in the presence of cells (LDL+cell) or LDL in the presence of cells and different concentrations of thyroid compounds (LDL+cell+compound) after 24 h of macrophage-induced LDL oxidation. Values are means  $\pm$  S.E.M. from four to six separate experiments performed in triplicate. In each experiment, each result was corrected by its control without LDL, then by its control without cells (except for LDL alone). For LDL and LDL+cell, the different values were obtained with thyroid compound diluent concentrations similar to those used in the presence of thyroid compounds, i.e. with NaOH concentrations ranging from 0.0025% to 0.1% (see Materials and Methods for details).

of the compounds, whereas it was less decreased by  $T_3$  and  $TA_3$ , reaching 5–7 nmol/mg protein at 20  $\mu$ M. Compound concentrations which decreased TBARS production to 50% ( $IC_{50}$ ) are shown in Table 1;  $T_3$  and  $TA_3$  had similar  $IC_{50}$  values of about 10  $\mu$ M whereas  $T_4$

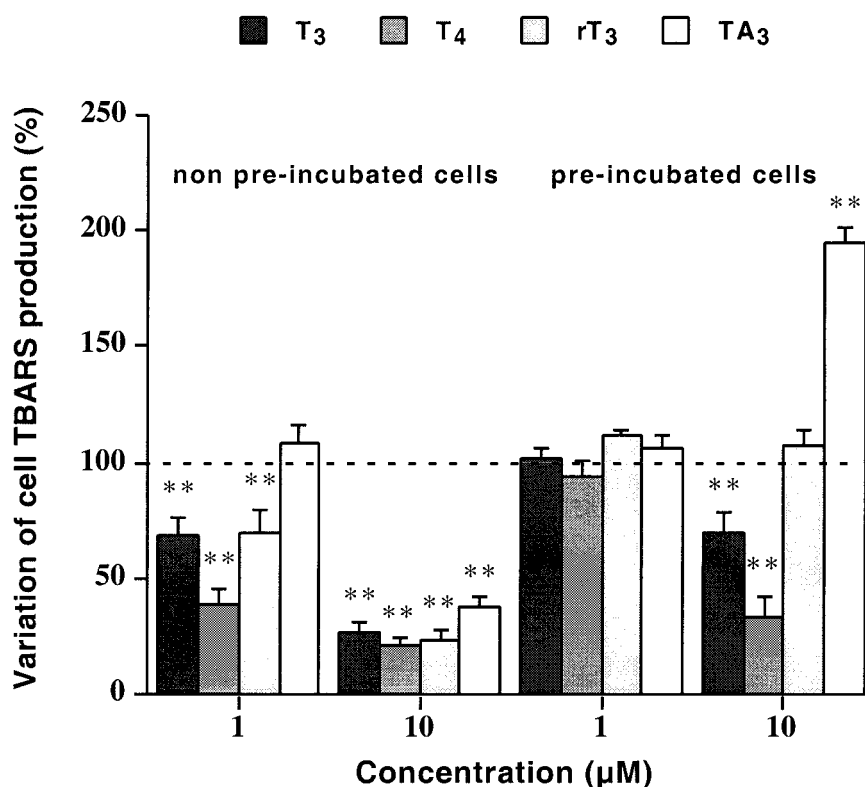
**Table 1** Thyroid compound concentrations ( $\mu$ M) which decreased TBARS production to 50% ( $IC_{50}$ ) during 24 h macrophage-induced LDL oxidation. Values are means  $\pm$  S.E.M. from four to six data sets calculated from logarithmic regressions established in separate experiments performed in triplicate

$T_3$	$T_4$	$rT_3$	$TA_3$
11.0 $\pm$ 2.6	1.4 $\pm$ 0.5**	0.9 $\pm$ 0.3**	8.1 $\pm$ 0.8

\*\* $P < 0.01$  compared with  $T_3$  (Dunnnett's test).

and  $rT_3$  had an  $IC_{50}$  of about 1  $\mu$ M. Thus, these two concentrations were used in further experiments.

The following results were obtained with macrophages preincubated or not with the thyroid compounds. When oxidation experiments were performed without LDL (Fig. 2), TBARS released in the incubation medium corresponded to the cell lipid peroxidation. When the cells were not preincubated, all the compounds at 10  $\mu$ M substantially decreased TBARS released by the cells. As expected, the decrease was less important with 1  $\mu$ M of the compound, except for  $TA_3$  which did not modify cell TBARS production. When the cells were preincubated with the compounds at 1  $\mu$ M, TBARS release was not modified by any compound. However, at 10  $\mu$ M,  $T_3$  and especially  $T_4$  protected macrophages from oxidation whereas  $TA_3$  increased cell oxidation,  $rT_3$  having no effect.



**Figure 2** Variation percentages of cell TBARS production by macrophages preincubated or not in the presence of 1 or 10  $\mu\text{M}$  of different thyroid compounds during oxidation experiments without LDL. Values are means  $\pm$  s.e.m. from 15 data sets (five separate experiments performed in triplicate). \*\* $P < 0.01$  compared with the control without thyroid compound (100% TBARS production) (Student's *t*-test). Preincubated cells were incubated for 24 h with a thyroid compound, then washed and again incubated for 24 h. Non-preincubated cells were incubated for 24 h without any compound, then washed and again incubated for 24 h with a thyroid compound (see Materials and Methods for details).

Variation of TBARS production by LDL oxidized by macrophages preincubated or not with the compounds at 1 or 10  $\mu\text{M}$  is presented in Fig. 3. In the non-preincubated cells, all the compounds inhibited LDL TBARS production, the inhibition being higher at 10  $\mu\text{M}$  than at 1  $\mu\text{M}$  and more important for T<sub>4</sub> and rT<sub>3</sub> than for T<sub>3</sub> and TA<sub>3</sub>, confirming the previous results (Fig. 1). T<sub>3</sub> was the less effective compound whatever the concentration. In the preincubated cells, T<sub>4</sub> and TA<sub>3</sub> reduced LDL TBARS production slightly at 1  $\mu\text{M}$  and by about 70% and 50% at 10  $\mu\text{M}$  respectively. T<sub>3</sub> and rT<sub>3</sub> had no effect at 1  $\mu\text{M}$  and decreased LDL TBARS only by about 10% at 10  $\mu\text{M}$ .

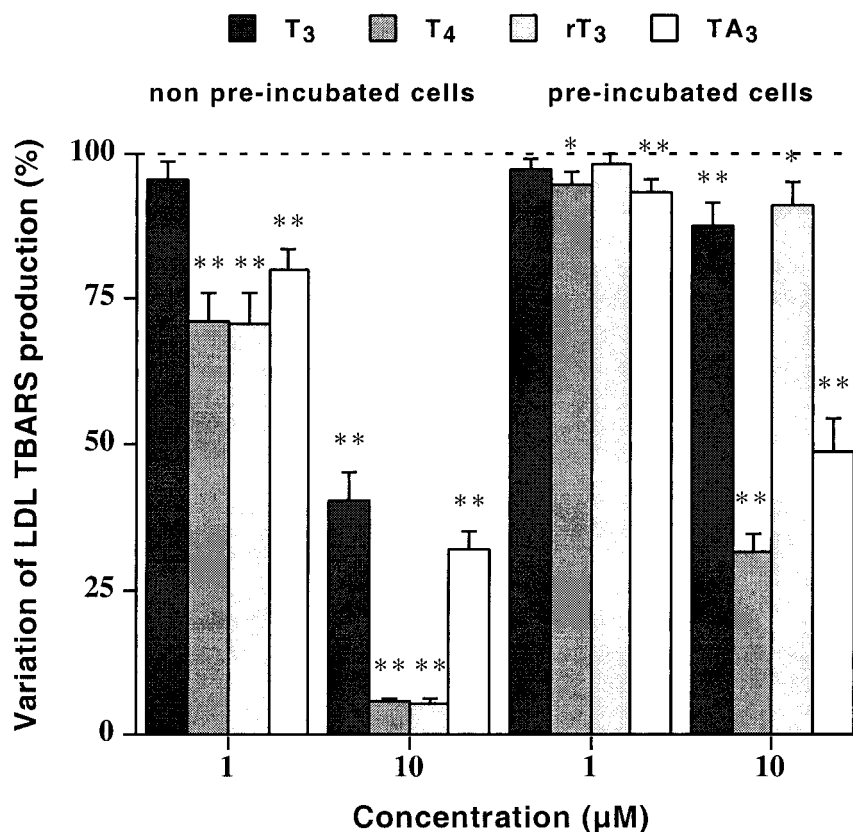
Table 2 gives cell viability percentages assessed by LDH activity measurements during oxidation experiments with or without LDL. In the absence of thyroid compounds (control), cell viability in the presence of LDL was somewhat lower than that in the absence of LDL (one-way ANOVA,  $P = 0.07$ ), indicating that oxidized LDL were slightly toxic for macrophages. T<sub>3</sub>, T<sub>4</sub> or rT<sub>3</sub> preincubated or not with macrophages did not change cell viability. On

the contrary, TA<sub>3</sub> lowered cell viability as compared with the control, especially at the 10  $\mu\text{M}$  concentration in macrophages preincubated or not with it, in the presence or in the absence of LDL (one-way ANOVA,  $P < 0.01$ ).

## Discussion

Culture of monocytic U937 cells and their differentiation into macrophages was performed in RPMI-1640 medium, whereas oxidation experiments were carried out in Ham's F-10 medium supplemented with Fe<sup>2+</sup>. Indeed, as reported by others, we have checked in preliminary studies that (1) very little if any macrophage LDL oxidation occurred in RPMI-1640 supplemented with up to 10  $\mu\text{M}$  Fe<sup>2+</sup> (Müller *et al.* 1998, Van Reyk *et al.* 1999), (2) iron was necessary to catalyze macrophage-mediated LDL oxidation (Müller *et al.* 1998, Yuan & Brunk 1998), and (3) Ham's F-10 medium containing 3  $\mu\text{M}$  Fe<sup>2+</sup> ought to be supplemented with an additional 5  $\mu\text{M}$  Fe<sup>2+</sup> in order to





**Figure 3** Variation percentages of LDL TBARS production by macrophages preincubated or not in the presence of 1 or 10  $\mu\text{M}$  of different thyroid compounds during oxidation experiments with LDL. Values are means  $\pm$  S.E.M. from 15 data sets (five separate experiments performed in triplicate). \* $P < 0.05$  and \*\* $P < 0.01$  compared with the control without thyroid compound (100% TBARS production) (Student's *t*-test). Preincubated cells were incubated for 24 h with a thyroid compound, then washed and again incubated for 24 h. Non-preincubated cells were incubated for 24 h without any compound, then washed and again incubated for 24 h with a thyroid compound. In each experiment, each data set was corrected by its control without LDL (see Materials and Methods for details).

avoid limitation of LDL oxidation which may occur in some LDL preparations (Marchant *et al.* 1996). Note that this  $\text{Fe}^{2+}$  concentration induced neither LDL peroxidation in the absence of cells (Fig. 1) nor cell toxicity (Table 2), and that such iron concentrations have been reported in human atherosclerotic lesions (Yuan *et al.* 1996).

Thyroid hormones and structural analogues were reported to protect LDL from oxidation induced by non-cellular pro-oxidant agents (Hanna *et al.* 1993, Chomard *et al.* 1998, Oziol *et al.* 2001) and by endothelial cells (Hanna *et al.* 1995). In the present study, we found that these compounds also reduced macrophage-induced LDL oxidation in a concentration-dependent manner. Furthermore, they also protected macrophages themselves from lipid peroxidation. Such an observation was foreseeable since analogues of thyroid hormones with a 4'-hydroxy diphenylether structure have free radical scavenging capacity (Oziol *et al.* 2001) and since macrophages are

able to generate ROS (Chisolm *et al.* 1999, Kaplan & Aviram 1999) which may be captured by the thyroid compounds. The ability of thyroid compounds to decrease *in vitro* LDL oxidation induced by non-cellular pro-oxidant systems is a physico-chemical property of these compounds which may be effective whatever the pro-oxidant system.

However,  $\text{T}_0$ , which has a great antioxidant effect on  $\text{Cu}^{2+}$ - or AAPH-induced LDL oxidation (Chomard *et al.* 1998, Oziol *et al.* 2001), was unable to protect LDL against macrophage-induced oxidation. This surprising observation led us to measure the antioxidant ability of the compounds in a classical *in vitro* LDL oxidation system, using Ham's F-10 medium as the working buffer. LDL were oxidized by  $\text{Cu}^{2+}$  or AAPH in the presence or not of thyroid compounds and conjugated dienes were continuously measured as previously described (Chomard *et al.* 1998). The results showed that Ham's F-10 itself protected

**Table 2** Viability (%) of macrophages preincubated or not in the presence of 1 or 10  $\mu\text{M}$  of different thyroid compounds during oxidation experiments with or without LDL. Values are means  $\pm$  s.e.m. from four separate experiments performed in triplicate

		Control	T <sub>3</sub>	T <sub>4</sub>	rT <sub>3</sub>	TA <sub>3</sub>
Without LDL		( $\mu\text{M}$ )				
Non-preincubated cells	1	102 $\pm$ 3	101 $\pm$ 1	103 $\pm$ 2	103 $\pm$ 5	100 $\pm$ 2
	10	99 $\pm$ 1	105 $\pm$ 2	98 $\pm$ 1	106 $\pm$ 3	90 $\pm$ 5
Preincubated cells	1	102 $\pm$ 4	104 $\pm$ 4	103 $\pm$ 3	104 $\pm$ 3	101 $\pm$ 3
	10	102 $\pm$ 1	102 $\pm$ 3	97 $\pm$ 1	100 $\pm$ 1	91 $\pm$ 6
With LDL		( $\mu\text{M}$ )				
Non-preincubated cells	1	96 $\pm$ 4	100 $\pm$ 2	103 $\pm$ 3	104 $\pm$ 3	101 $\pm$ 3
	10	98 $\pm$ 4	105 $\pm$ 2	101 $\pm$ 2	107 $\pm$ 4	96 $\pm$ 3
Preincubated cells	1	98 $\pm$ 4	102 $\pm$ 4	102 $\pm$ 4	99 $\pm$ 5	100 $\pm$ 4
	10	99 $\pm$ 5	102 $\pm$ 3	100 $\pm$ 1	100 $\pm$ 3	88 $\pm$ 5

Preincubated cells were incubated for 24 h with a thyroid compound, then washed and again incubated for 24 h. Non-preincubated cells were incubated for 24 h without any compound, then washed and again incubated for 24 h with a thyroid compound. For control, the values were obtained with thyroid compound diluent concentrations similar to those used in the presence of thyroid compounds, i.e. with 0.005% and 0.05% NaOH (see Materials and Methods for details).

LDL against oxidation by the pro-oxidant agents, as compared with similar experiments performed in PBS, and that thyroid compounds had practically no additional antioxidant effects in the Ham's F-10 medium, particularly T<sub>0</sub> (data not shown). The composition of Ham's F-10, rich in sulphured amino acids like cysteine or methionine and in vitamins like thiamine or pyridoxine, may well explain its antioxidant properties. In the present work using Ham's F-10 as oxidation medium, T<sub>0</sub> was antioxidant inactive whereas other thyroid compounds were antioxidant active. It then appears that the physico-chemical antioxidant property of thyroid compounds was not the major mechanism responsible for the protection of LDL against macrophage oxidation, at least in the extracellular medium.

Macrophages may oxidize extracellular LDL by releasing ROS generated via different enzymatic systems or derived from cell lipid peroxidation (Chisolm *et al.* 1999, Kaplan & Aviram 1999). Transition metal ions like Fe<sup>2+</sup> may favour macrophage ROS generation and LDL radical attack (Xing *et al.* 1998, Yuan & Brunk 1998). If the radical scavenging capacity of thyroid compounds was predominant outside the cells, T<sub>3</sub> and TA<sub>3</sub> ought to have decreased LDL lipid peroxidation more than T<sub>4</sub> and rT<sub>3</sub>, as previously found during AAPH-induced LDL oxidation (Oziol *et al.* 2001); however, we found the contrary here. Since T<sub>0</sub> had no antioxidant effect here and considering the antioxidant capacity of Ham's F-10, as discussed above, it may be proposed that T<sub>0</sub> does not enter the macrophages whereas the other compounds do, and/or that these compounds act on macrophage cellular mechanisms whereas T<sub>0</sub> does not. Consistent with this hypothesis is that T<sub>4</sub> and T<sub>3</sub>, but not T<sub>0</sub>, inhibit lipid peroxidation in rat liver mitochondria (Cash *et al.* 1967); T<sub>0</sub>, which has no iodine, is less lipophilic than the iodo-thyroid compounds

and probably cannot enter the cells by passive diffusion as do other iodothyronines (Hulbert 2000); finally, the iodo compounds are also able to enter cells by an energy-dependent transport system located in the plasma membrane (Kragie 1994, 1996) and are present in macrophages (Liu *et al.* 1989).

Thyroid compounds are able to act by several mechanisms inside the macrophage. As free radical scavengers (Oziol *et al.* 2001), they may reduce cellular flux of ROS, perhaps degrading themselves by ether-link cleavage. Indeed, this is a thyroid hormone oxidation process, frequently observed during *in vitro* experiments using macrophages and which needs H<sub>2</sub>O<sub>2</sub> (Green 1994). This process would be (1) a major pathway of thyroid hormone degradation into human phagocytes, (2) favoured by different proteins with peroxidase activity like myeloperoxidase, and (3) active on T<sub>4</sub> as well as on T<sub>3</sub> (Klebanoff & Green 1973, Burger *et al.* 1983, Meinhold & Buchholz 1986). Thus ether-link cleavage of iodothyronines could consume H<sub>2</sub>O<sub>2</sub> and reduce the activity of macrophage myeloperoxidase which is involved in LDL Apolipoprotein B100 and lipid oxidation (Carr *et al.* 2000). Thyroid compounds may also chelate cell transition metals. Indeed, T<sub>3</sub> and TA<sub>3</sub> have been shown to disturb LDL Cu<sup>2+</sup> binding suggesting that they have chelating properties for this metal (Faure *et al.* 1999).

Free radical scavenging or transition metal chelation do not completely explain our results, particularly when macrophages were preincubated with the thyroid compounds, then rinsed, then incubated again with native LDL. First, T<sub>3</sub> and TA<sub>3</sub> reduced, more severely than T<sub>4</sub> and rT<sub>3</sub>, LDL oxidation induced by Cu<sup>2+</sup> (Chomard *et al.* 1998) or AAPH (Oziol *et al.* 2001), whereas we found the contrary here in the non-preincubated macrophages. Secondly, in the preincubated macrophages, T<sub>4</sub> protected

the cells (Fig. 2) and the LDL (Fig. 3) from oxidation, much more than  $T_3$ . We can then assume that thyroid compounds act partly on different systems responsible for the oxidative status of cells.

High affinity  $T_3$  and  $T_4$  receptors exist in human mononuclear cells (Burman *et al.* 1980) and thyroid hormones are known to regulate redox enzyme activities. During hyperthyroidism, NAD(P)H oxidase activity was increased in rat (Fernandez & Videla 1995) and human (Magsino *et al.* 2000) leukocytes or human phagocytes (Videla *et al.* 1993); this was also true for Cu/Zn- and Mn-superoxide dismutase (SOD) in rat leukocytes (Pereira *et al.* 1994). In hypothyroid rat macrophages, Mn-SOD, catalase and glutathion peroxidase (GPx) activities were decreased; thyroid hormones added to cultured rat macrophages increased SOD activities, leading to a decrease in cell  $H_2O_2$  production and GPx activity (Pereira *et al.* 1995). Myeloperoxidase was stimulated by  $T_3$  and  $T_4$  in isolated human leukocytes (Van Zyl *et al.* 1989). Thus, thyroid compounds are susceptible to induce modifications in the macrophage oxidant/antioxidant status, the result of which determines their overall cellular redox effect. In the preincubated cells, the modifications of lipid peroxidation may be partly due to the effect of thyroid compounds on pro- or antioxidant enzyme expression, whereas in the non-preincubated cells, physico-chemical antioxidant properties of the compounds may be modulated by this probably transcriptional effect. This hypothesis is supported by the fact that  $rT_3$ , which has no affinity for thyroid hormone receptors (Jorgensen 1981), had practically no more activity in the preincubated cells, either on lipid peroxidation of macrophages (Fig. 2) or on that of LDL (Fig. 3).

Surprisingly,  $TA_3$  protected LDL from oxidation in the non-preincubated cells as  $T_3$  did (Figs 1 and 3), whereas  $TA_3$  was more effective than  $T_3$  in the preincubated cells (Fig. 3). It may be suspected that  $TA_3$ , which has a higher thyroid hormone receptor affinity than  $T_3$  (Takeda *et al.* 1995), behaves differently from  $T_3$  on the redox systems. However,  $TA_3$  slightly decreased macrophage viability whereas the other compounds did not (Table 2). It also protected cells from peroxidation less than the other compounds, particularly at  $10 \mu M$  in the preincubated cells where it increased TBARS production (Fig. 2). As a result, the rather significant inhibition of LDL lipid peroxidation by  $TA_3$  (Fig. 3) may be explained, at least in part, by (1) a partial destruction of cells which reduce their pro-oxidant capabilities (Marchant *et al.* 1996, Müller *et al.* 1998), and (2) an artefact due to the calculation of this inhibition, i.e. subtraction of TBARS due to cell lipid peroxidation from those due to cell and LDL lipid peroxidation. Indeed, TBARS from cells being high with  $TA_3$  (at  $10 \mu M$ :  $9.4 \text{ nmol}$  versus less than  $5.4 \text{ nmol}$  in all other cases), their subtraction from cell and LDL TBARS undervalued LDL TBARS production, leading to an underestimation of the variation percentage.

Finally, thyroid compounds might act as antioxidants in cell membranes (Hulbert 2000). In the rat,  $T_4$  content of different tissues is proportional to the phospholipid content of these tissues. Thyroid hormones rigidify membranes with which they combine, reducing their fluidity; it is a very fast phenomenon, independent of the classical action of hormones, and  $T_3$  and  $T_4$  may have different effects. The direct antioxidant role of thyroid compounds inside biological membranes remains to be proved. It should, however, be taken into account for the interpretation of our results concerning  $rT_3$ . Indeed, this compound revealed a great antioxidant effect against macrophage-induced LDL oxidation in the non-preincubated cells, as high as that of  $T_4$  (Figs 1 and 3), whereas it is thyromimetic inactive and was as antioxidant as  $T_4$ , and less antioxidant than  $T_3$  during LDL oxidation by non-cellular pro-oxidant agents (Chomard *et al.* 1998, OzioU *et al.* 2001). Note that  $rT_3$ , like  $T_4$ , possesses two iodine atoms in the 3' and 5' position which influence their lipophilicity (Hulbert 2000), whereas  $T_3$  and  $TA_3$  have only one iodine atom at 3'. The fact that  $rT_3$  has *a priori* no effect on thyroid hormone receptors cannot explain by itself its great antioxidant effect here, as compared with  $T_4$ .

In conclusion, during macrophage-induced LDL oxidation, the thyroid compounds probably act by different mechanisms which explains the differences observed between them. They have physico-chemical antioxidant effects which was demonstrated in  $Cu^{2+}$ - and AAPH-induced LDL oxidation *in vitro* (Hanna *et al.* 1993, Chomard *et al.* 1998, OzioU *et al.* 2001), and cellular effects due to their binding on the thyroid hormone receptors and probably to other non-transcriptional effects. The more significant result of our work is that overall cellular effects of  $T_3$  led to a reduction of its antioxidant capacity, whereas those of  $T_4$  induced an increase in its ability to reduce macrophage-induced LDL oxidation. Thus  $T_4$  might have a protective effect against cellular-induced LDL oxidation *in vivo*. This hypothesis is consistent with the fact that LDL are more oxidizable and more oxidized in hypothyroid than in euthyroid subjects, perturbations which are restored by a  $T_4$  treatment (Sundaram *et al.* 1997, Diekman *et al.* 1998).

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