The Journal of Experimental Biology 209, 817-825
Published by The Company of Biologists 2006
doi:10.1242/jeb.02045

Differential effects of experimental and cold-induced hyperthyroidism on factors inducing rat liver oxidative damage

P. Venditti[1,*], R. Pamplona[2], V. Ayala[2], R. De Rosa[1], G. Caldarone[1] and S. Di Meo[1]

[1]*Dipartimento delle Scienze Biologiche, Sezione di Fisiologia, Università di Napoli, I-80134 Napoli, Italy and*
[2]*Departament de Ciènces Mèdiques Bàsiques, Universitat de Lleida, 25198 Lleida, Spain*

*Author for correspondence (e-mail: venditti@unina.it)

Accepted 19 December 2005

Summary

Thyroid hormone-induced increase in metabolic rates is often associated with increased oxidative stress. The aim of the present study was to investigate the contribution of iodothyronines to liver oxidative stress in the functional hyperthyroidism elicited by cold, using as models cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated rats. The hyperthyroid state was always associated with increases in both oxidative capacity and oxidative damage of the tissue. The most extensive damage to lipids and proteins was found in T_3-treated and cold-exposed rats, respectively. Increase in oxygen reactive species released by mitochondria and microsomes was found to contribute to tissue oxidative damage, whereas the determination of single antioxidants did not provide information about the possible contribution of a reduced effectiveness of the antioxidant defence system. Indeed, liver oxidative damage in hyperthyroid rats was scarcely related to levels of the liposoluble antioxidants and activities of antioxidant enzymes. Conversely, other biochemical changes, such as the degree of fatty acid unsaturation and hemoprotein content, appeared to predispose hepatic tissue to oxidative damage associated with oxidative challenge elicited by hyperthyroid state.

As a whole, our results confirm the idea that T_3 plays a key role in metabolic changes and oxidative damage found in cold liver. However, only data concerning changes in glutathione peroxidase activity and mitochondrial protein content favour the idea that dissimilarities in effects of cold exposure and T_3 treatment could depend on differences in serum levels of T_4.

Key words: cold exposure, antioxidant capacity, free radical, lipid peroxidation, protein carbonyl.

Introduction

It is well documented that a hypermetabolic state induced by thyroid hormone administration to experimental animals is accompanied by oxidative stress in several target tissues (Videla, 2000). Studies on liver, heart and muscle suggest that such oxidative stress is due to increased mitochondrial release of reactive oxygen species (ROS) (Fernández and Videla, 1993; Venditti et al., 2003a; Venditti et al., 2003b; Venditti et al., 2003c) and reduced global efficacy of the antioxidant defence system (Venditti et al., 1997; Videla, 2000).

Hyperthyroid state can also be induced in homeothermic animals by physiological modifications of thyroid activity. In rat, exposure to a cold environment is associated with increased serum 3,5,3′-triiodothyronine (T_3) levels (Nejad, 1972), which is thought to be the main factor responsible for the heat produced by non-shivering thermogenesis (Jansky, 1963). Although in rodents the changes in metabolic activity involved in such process occur mostly in brown adipose tissue (BAT) (Himms-Hagen, 1983), they are also found in other tissues, including liver (Goglia et al., 1983), and cardiac (Venditti et al., 2003b) and skeletal (Guernsey and Stevens, 1977) muscles.

The effects of cold-induced hyperthyroidism on tissue oxidative damage have been scarcely investigated. However, indications have been obtained for an increase in lipid peroxidation products in several rat tissues, including BAT (Barja de Quiroga et al., 1991), lung (Tnimov et al., 1984), muscle (Venditti et al., 2004a), liver and heart (Venditti et al., 2004a; Kolosova et al., 1995). Furthermore, it has been recently reported that the liver from 10-day cold-exposed rats exhibits increases in ROS released by mitochondria (Venditti et al., 2004b) and susceptibility to oxidative challenge (Venditti et al., 2004a).

Because such modifications are similar to those induced in the liver by T_3 administration, we proposed that this hormone brings about the biochemical changes underlying tissue oxidative damage found in the two conditions (Venditti et al., 2004a; Venditti et al., 2004b). However, unlike cold exposure, T_3 administration strongly decreases serum levels of thyroxine (T_4), which has been reported to have intrinsic biological activity in

the cold (Cageao et al., 1992). Thus, it is possible that T_4 contributes to some changes underlying tissue thermogenesis and oxidative stress associated with cold exposure. If so, differences should have to be found in the tissue responses to treatments which differentially affects circulating T_4 levels.

Therefore, we compared the effects of 10 days of thyroid hormone (T_3 or T_4) treatment and cold exposure on oxidative metabolism and the extent of oxidative damage in rat liver. To obtain information on oxidative damage to lipids and proteins we measured levels of hydroperoxides and protein-bound carbonyls, respectively. Furthermore, we measured levels of markers of non-enzymatic protein modifications that form under oxidative conditions.

Mitochondrial and microsomal ROS production and parameters determining the susceptibility to oxidative challenge were also assessed. For this purpose, glutathione peroxidase (GPX) and glutathione reductase (GR) activities, vitamin E (Vit E), coenzyme Q (CoQ), and reduced glutathione (GSH) content, lipid composition, and response to oxidants *in vitro* of the tissues were determined.

Materials and methods

Materials

All chemicals used (Sigma Chimica, Milano, Italy) were of the highest grades available. Response to oxidative stress was determined by using reagents and instrumentation of the commercially available Amerlite System (Ortho-Clinical Diagnostics, Milano, Italy). Serum levels of free triiodothyronine (FT_3) and thyroxine (FT_4) were determined by using commercial RIA kits (DiaSorin, Salluggia, Italy).

Animals

The experiments were carried out on 60-day-old male Wistar rats (*Rattus norvegicus albius* Berkenhault 1769), supplied by Nossan (Correzzana, Italy) at day 45 of age. From day 50, animals were randomly assigned to one of four groups: euthyroid control rats (C), and rats made hyperthyroid by T_3 (HT_3) or T_4 (HT_4) treatment (10 days of daily intraperitoneal injections of 10 μg 100 g^{-1} body mass of T_3 or T_4, respectively) or by cold exposure (10 days at 4±1°C) (CE). C, HT_3 and HT_4 rats were kept at room temperature of 24±1°C. All rats were subjected to the same conditions (one per cage, constant artificial circadian cycle of 12 h:12 h L:D, and 50±10% relative humidity), and fed the same diet of a commercial rat chow purchased from Nossan, and water on an *ad libitum* basis.

The treatment of animals in these experiments was in accordance with the guidelines set forth by the University's Animal Care Review Committee.

Tissue preparation

The animals were sacrificed by decapitation while under ether anaesthesia. Arterial blood samples were collected and later analysed to determine plasma levels of FT_3 and FT_4 by radioimmunoassay. Liver was rapidly excised and placed into ice-cold homogenisation medium (HM) (220 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 0.1% fatty acid-free albumin, 10 mmol l^{-1} Tris, pH 7.4). Then, the tissue was weighed, finely minced and washed with HM. Finally, liver fragments were gently homogenised (20% w:v) in HM using a glass Potter-Elvehjem homogeniser set at a standard velocity (500 r.p.m.) for 1 min. Aliquots of liver homogenates were used for analytical procedures and preparation of mitochondrial and microsomal fractions.

Preparation of mitochondrial and microsomal fractions

The homogenates, diluted 1:1 with HM, were freed of debris and nuclei by centrifugation at 500 ***g*** for 10 min at 4°C. The resulting supernatants were centrifuged at 10 000 ***g*** for 10 min. The mitochondrial pellets were washed twice with isolation medium (IM; 220 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, 1 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris, pH 7.4), resuspended in the same solution and used for determination of cytochrome oxidase (COX) activity and H_2O_2 release. The 10 000 ***g*** supernatants were centrifuged at 105 000 ***g*** for 60 min and the resulting microsomal pellets were suspended in IM and used for determination of glucose-6-phosphatase activity and H_2O_2 production.

The protein content of liver preparations was determined, after solubilization in 0.5% deoxycholate, by the biuret method (Gornall et al., 1949) with bovine serum albumin as standard.

Cytochrome oxidase and glucose-6-phosphatase activities

Cytochrome oxidase activity of homogenates and mitochondrial suspensions was determined by the procedure of Barré et al. (Barré et al., 1987). Glucose-6-phosphatase activity was determined in liver homogenates and microsomal preparations as described by Katewa and Katyare (Katewa and Katyare, 2003).

Liver oxygen consumption

Liver oxygen consumption was monitored at 30°C by a Gilson respirometer in 1.6 ml of incubation medium (145 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 5 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EGTA, pH 7.4) with 50 μl of homogenate and succinate (10 mmol l^{-1}), plus 5 μmol l^{-1} rotenone (Rot), or pyruvate/malate (10/2.5 mmol l^{-1}) as substrates, in the absence (state 4) and in the presence (state 3) of 500 μmol l^{-1} ADP.

Oxidative damage to lipids and proteins

The extent of the peroxidative processes in tissue homogenates was determined by measuring the level of lipid hydroperoxides according to the method of Heath and Tappel (Heath and Tappel, 1976). Tissue protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls as described by Reznick and Packer (Reznick and Packer, 1994).

Concentrations of markers of non-enzymatic protein modifications, such as glutamic (GSA) and aminoapidic semialdehyde (AASA) (resulting from direct protein

oxidation), N^{ϵ}-(carboxymethyl)lysine (CML) (resulting from both lipid peroxidation and glycoxidation), N^{ϵ}-(carboxyethyl)lysine (CEL) (resulting from glycoxidative damage), and N^{ϵ}-(malondialdehyde)lysine (resulting from malondialdehyde attachment to protein lysine residue) were detected and measured by gas chromatography/mass spectrometry (GC/MS) as previously described (Pamplona et al., 2005).

Mitochondrial and microsomal H_2O_2 release

The rate of mitochondrial and microsomal H_2O_2 release was measured at 30°C following the increase in fluorescence (excitation at 320 nm, emission at 400 nm) resulting from oxidation of *p*-hydroxyphenylacetate (PHPA) by H_2O_2 in the presence of horseradish peroxidase (HRP) (Hyslop and Sklar, 1984) in a computer-controlled Jasko fluorometer equipped with a thermostatically controlled cell holder. For measurement of H_2O_2 produced by the respiratory chain, the reaction mixture consisted of 0.1 mg ml^{-1} mitochondrial proteins, 6 U ml^{-1} HRP, 200 μg ml^{-1} PHPA and 10 mmol l^{-1} succinate, plus 5 μmol l^{-1} rotenone, or 10 mmol l^{-1} pyruvate/2.5 mmol l^{-1} malate added last to start the reaction in a medium containing 145 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 5 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EGTA, pH 7.4. Measurements with the different substrates in the presence of 500 μmol l^{-1} ADP were also performed. For measurement of H_2O_2 produced by monoamine oxidase (MAO) the reaction mixture consisted of 0.1 mg ml^{-1} mitochondrial proteins, 6 U ml^{-1} HRP, 200 μg ml^{-1} PHPA and 0.2 mmol l^{-1} tyramine added last to start the reaction in the same medium used for respiration-linked H_2O_2 release. For microsomal preparations the reaction mixture consisted of 0.422 mg ml^{-1} microsomal proteins, 6 U ml^{-1} HRP, 200 μg ml^{-1} PHPA in 0.1 mol l^{-1} phosphate buffer, pH 7.4. The H_2O_2 produced was determined by the fluorescence change 10 min after addition of 0.35 mol l^{-1} NADPH.

Known concentrations of H_2O_2 were used to establish the standard concentration curve. Preliminary experiments studied the effect of catalase addition on the measured rates of H_2O_2 production. They showed a dose-dependent drop of the fluorescence in the presence of the enzyme.

Activities of antioxidant enzymes and levels of low-molecular mass antioxidants

Liver GPX activity was assayed at 37°C according to the method of Flohé and Günzler (Flohé and Günzler, 1984), with H_2O_2 as substrate. GR activity was measured at 30°C according to Carlberg and Mannervik (Carlberg and Mannervik, 1985).

Ubiquinols ($CoQH_2$) from 0.5 ml of 10% homogenate were oxidized to ubiquinones (CoQs) with 0.5 ml of 2% $FeCl_3$ and 2.0 ml of ethanol. The total content of CoQs ($CoQH_2$ + CoQ) was then determined as described by Lang et al. (1986). Vit E content was determined using the HPLC procedure of Lang et al. (Lang et al., 1986). GSH concentration was measured as described by Griffith (Griffith, 1980).

Lipid composition

Fatty acyl groups were analysed by GC/MS as previously described (Pamplona et al., 1998). Fatty acyl composition of lipids was expressed as mol%.

The following fatty acyl indices were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL)=[(Σ%$Total_{14}$×14)+(Σ%$Total_{16}$×16)+(Σ%$Total_{18}$×18)+(Σ%$Total_{20}$×20)+(Σ%$Total_{22}$×22)]/100; double bond index (DBI)=[(1×Σmol% monoenoic)+(2×Σmol% dienoic)+(3×Σmol% trienoic)+(4×Σmol% tetraenoic)+(5×Σmol% pentaenoic)+(6×Σmol% hexaenoic)], and peroxidizability index (PI)=[(0.025×Σmol% monoenoic)+(1×Σmol% dienoic)+(2×Σmol% trienoic)+(4×Σmol% tetraenoic)+(6×Σmol% pentaenoic)+(8×Σmol% hexaenoic)].

Susceptibility to oxidative challenge

Response to oxidative challenge was determined as previously described (Venditti et al., 1999). Briefly, samples of 10% (w:v) homogenates were obtained by diluting the 20% homogenates with equal volumes of 0.2% Lubrol in 15 mmol l^{-1} Tris, pH 8.5. Several dilutions of samples up to a tissue concentration of 0.002% were prepared in 15 mmol l^{-1} Tris (pH 8.5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were initiated by addition of 250 μl of the reaction mixture to 25 μl of the samples. The plates were incubated at 37°C for 30 s with continuous shaking and then transferred to a luminescence analyser. The emission values were fitted to dose-response curves using the statistical facilities of the Fig.P graphic program (Biosoft, Cambridge, UK).

Data analysis

The data, expressed as means ± standard error, were analyzed with a one-way analysis of variance method (ANOVA). When a significant *F* ratio was found, the Student-Newman–Keuls multiple range test was used to determine the statistical significance between means. Probability values (P)<0.05 were considered significant. In Fig. 2 the results of the experiments are presented as sample curves.

Results

Changes in thyroid state were documented by modifications in heart mass/body mass ratio, and plasma levels of FT_3 and FT_4. The body masses of C, CE, HT_3 and HT_4 rats, which were 267±4, 253±6, 251±6 and 252±8 g, respectively, were not significantly affected by any of the treatments. Conversely, the heart masses increased in all hyperthyroid rats so that heart/body mass ratios of these animals (3.25±0.05, 3.40±0.11 and 3.17±0.07 mg g^{-1} for CE, HT_3, and HT_4 rats, respectively) were higher than control values, without any significant difference among them. Plasma levels of FT_3 increased in all hyperthyroid rats, but

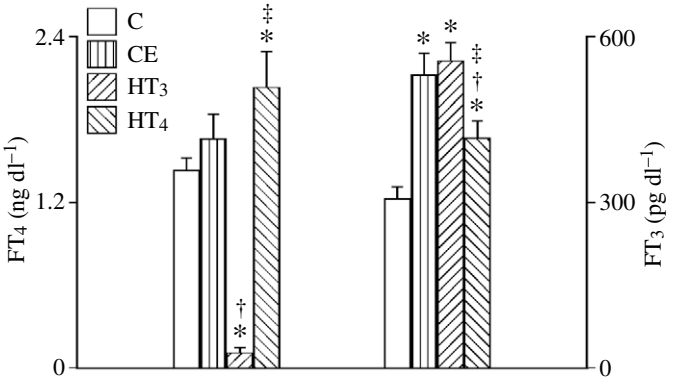

Fig. 1. Serum levels of free triodothyronine (FT_3) and thyroxine (FT_4) in control (C), cold-exposed (CE), and T_3-treated (HT_3) or T_3-treated (HT_4) rats. Values are means ± s.e.m. of eight different experiments. *Significant *vs* C rats; †significant *vs* CE rats; ‡significant *vs* HT_3 rats. The level of significance was chosen as $P<0.05$.

they were lower in HT_4 than in CE and HT_3 rats, whereas the FT_4 levels increased in CE and HT_4 rats and decreased in HT_3 rats (Fig. 1).

Cytochrome oxidase and glucose-6-phosphatase activities

Cold exposure and hormonal treatments were associated with increases in COX activities in both homogenates and mitochondria, which were lower in HT_4 rats (Table 1). Homogenate COX activities were not significantly different after cold exposure or T_3 treatment, whereas mitochondrial COX activities reached the highest value after T_3 treatment. The *in vitro* COX activity has been positively correlated to the maximal oxygen consumption (Simon and Robin, 1971) so that its changes provided information on effects of the treatments on aerobic metabolic capacity of the biological preparations.

Glucose-6-phosphatase activities of homogenates and microsomes increased in all hyperthyroid rats, but the highest activity in the homogenates was found in that from HT_3 rats (Table 1).

The ratio between the cytochrome oxidase activities of homogenates and mitochondria and that between glucose-6-phosphatase activities of homogenates and microsomes provided rough estimates of tissue content of mitochondrial and microsomal proteins, respectively.

Mitochondrial protein content was higher after cold exposure and T_4 treatment, but not after T_3 treatment, whereas microsomal protein content was not affected by hormonal treatments and cold exposure (Table 1).

Liver oxygen consumption

The rates of both succinate- and pyruvate/malate-supported oxygen consumption are reported in Fig. 2. Those supported by succinate were increased by all treatments and were higher in HT_3 than in CE group in state 4, and higher in HT_3 than in other two groups in state 4. Those supported by pyruvate/malate were increased in all hyperthyroid groups and, in state 3, reached the highest value in HT_3 group.

Oxidative damage to lipids and proteins

The levels of hydroperoxides and protein-bound carbonyls were higher in hyperthyroid than control rats (Fig. 3). The highest and lowest hydroperoxide levels were found in HT_3 and CE preparations, respectively, whereas the highest carbonyl levels were found in CE preparations.

Of the non-enzymatic protein modification markers, GSA levels were increased by cold exposure and T_3 treatment, whereas that increase did not reach significant levels after T_4 treatment. The steady-state levels of AASA increased significantly by all treatments, reaching the highest and lowest magnitudes in HT_3 and HT_4 groups, respectively. CEL and CML levels were increased only by T_3 treatment. MDAL levels increased in all treatment groups and reached the lowest value in HT_4 group (Table 2).

Mitochondrial and microsomal H_2O_2 release

The effect of cold exposure and T_3/T_4 treatment on H_2O_2 release by succinate (complex II-linked substrate)- and pyruvate/malate (complex I-linked substrates)-supplemented mitochondria are showed in Fig. 4. With complex II-linked

Table 1. *Enzyme activities and protein content in mitochondria and microsomes from control, cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated rat liver*

	Cytochrome oxidase		Glucose-6-phosphatase		Protein	
Group	Homogenate	Mitochondria	Homogenate	Microsomes	Mitochondria	Microsomes
C	69.9±0.7	0.94±0.04	17.8±0.3	0.34±0.01	73.3±3.2	52.7±2.5
CE	116.7±1.7*	1.25±0.02*	20.6±0.3*	0.41±0.01*	93.4±3.6*	50.0±1.4
HT_3	111.5±0.9*	1.43±0.04*,†	22.2±0.8*,†	0.42±0.01*	77.9±2.3†	51.8±1.8
HT_4	88.6±1.7*,†,‡	1.05±0.04*,†,‡	20.3±0.3*,‡	0.42±0.01*	84.4±2.2*,†	48.7±2.1

Cytochrome oxidase activity is expressed as μmol O min^{-1} g^{-1} for liver and μmol O min^{-1} g^{-1} protein for mitochondria. Glucose-6-phosphatase activity is expressed as μmol P_i min^{-1} g^{-1} for liver and μmol Pi min^{-1} mg^{-1} protein for microsomes. Mitochondrial and microsomal protein content is expressed as mg protein g^{-1} tissue.

Values are means ± s.e.m. For each value eight rats were used.

*Significant *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

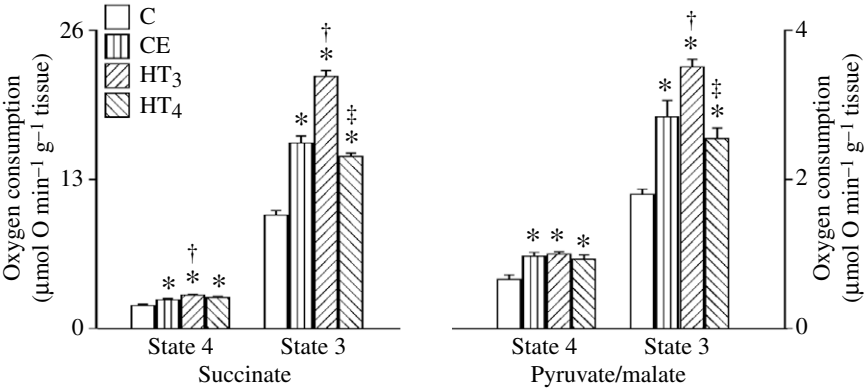

Fig. 2. Effect of cold exposure and hormonal [triodothyronine (T_3) or thyroxine (T_4)] treatment on oxygen consumption of liver homogenate. Values are means ± s.e.m. of eight different experiments. *Significant *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

substrate the rates of H_2O_2 release during state 4 respiration increased in all treatment groups and reached the greatest value in HT_3 group. During state 3 the rates increased only after hormonal treatments and reached the highest value in the HT_3 group. With complex I-linked substrates, during state 4, the rates significantly increased in all hyperthyroid groups and were greater in the HT_3 than in the CE group, whereas during state 3 they increased only in hormone-treated groups, reaching the highest value in the HT_4 group. The rates of H_2O_2 production by monoamine oxidase in the presence of tyramine were not significantly modified by cold exposure and hormonal treatment. Their values were 3.39±0.08, 3.35±0.17, 3.24±0.04, 3.49±0.12 nmol min^{-1} mg^{-1} protein for C, CE, HT_3, and HT_4 preparations, respectively. The rate of NADPH-dependent H_2O_2 production by liver microsomes, which was 119.1±3.7 pmol min^{-1} mg^{-1} protein in the C group, increased in all treatment groups. However, the value for the HT_3 group (219.4±8.7 pmol min^{-1} mg^{-1} protein) was significantly lower than those for the CE and HT_4 groups (259.7±6.4 and 247.5±10.6 pmol min^{-1} mg^{-1} protein, respectively).

Antioxidants

The content of both enzymatic and low molecular mass antioxidants in the liver are showed in Table 3. GPX activity was increased by T_3 and decreased by T_4 treatment, but was not affected by cold exposure. GR activity was increased by cold exposure and to a greater extent by T_3 treatment, whereas it was not modified by T_4 treatment. Vit E content was

Table 2. *Nonenzymatic protein modifications in liver from control, cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated rats*

Parameter	C	CE	HT_3	HT_4
GSA	3832±165	5007±167*	4567±125*	3986±195†,‡
AASA	131±4	185±3*	208±7*,†	158±7*,†,‡
CEL	369±15	345±13	407±5*,†	362±12‡
CML	938±34	1054±78	1077±45*	943±49‡
MDAL	144±4	198±5*	202±3*	177±12*,†,‡

Glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), N^{ϵ}-carboxymethyl-lysine (CML) and N^{ϵ}-carboxyethyl-lysine (CEL), and N^{ϵ}-malondialdehyde-lysine (MDAL) are expressed as μmol mol^{-1} lysine. Values are means ±s.e.m. For each value six rats were used.

*Significant ($P<0.05$) *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

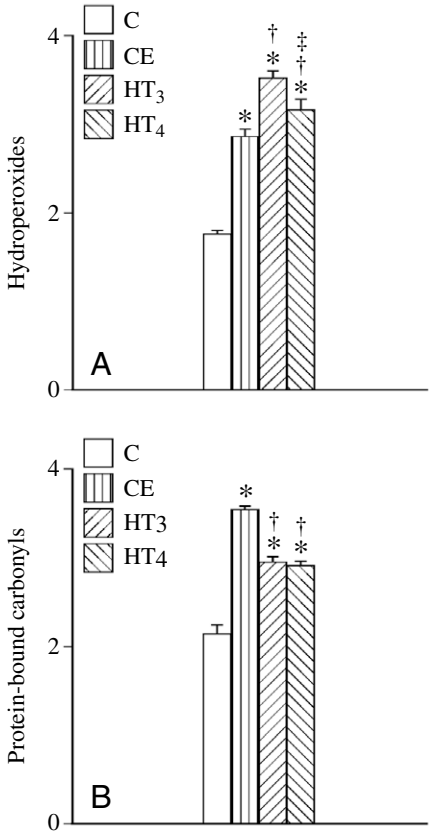

Fig. 3. Effect of cold exposure and hormonal [triodothyronine (T_3) or thyroxine (T_4)] treatment on oxidative damage of liver lipid and proteins. Hydroperoxides (A) are expressed in pmol NADP min^{-1} g^{-1} tissue; protein-bound carbonyls (B) are expressed in nmol mg^{-1} protein. Preparations were from control (C), 10-day cold exposed (CE), T_3-treated (HT_3), and T_4-treated (HT_4) rats. Values are means ± s.e.m. of eight different experiments. *Significant *vs* C rats; †significant *vs* CE rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

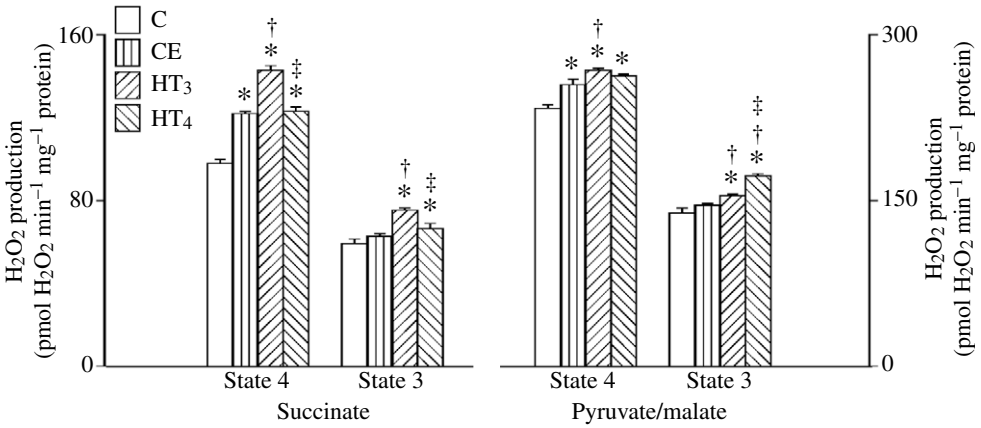

Fig. 4. Effect of cold exposure and triodothyronine (T_3) or thyroxine (T_4) treatment on H_2O_2 production by succinate and pyruvate/malate-supplemented mitochondria from rat liver. Values are means ± s.e.m. of eight different experiments. *Significant *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

increased by all treatments, reaching the highest value in the HT_3 group. CoQ9 levels were slightly increased by cold exposure, more strongly increased by T_3 treatment, and not modified by T_4 treatment, whereas CoQ10 levels were increased only by T_3 treatment. GSH levels were significantly reduced by all treatments.

Fatty acid composition

Effects of cold exposure and hormonal treatments on fatty acid profiles and indexes are shown in Table 4. Globally, the amount (%) of saturated fatty acids and unsaturated fatty acids is maintained constant among the different experimental groups. However, all experimental groups showed a change in the distribution of unsaturated fatty acids. Thus, cold exposure, T_3 and T_4 diminished the content of monounsaturated fatty acids and increased the content of polyunsaturated fatty acids (specially PUFA from the n-6 series). Those changes led to a significant increase in the double bond index and peroxidizability index in all hyperthyroid groups. The increase in PUFA n-6 was mainly due to the increase in the arachidonic acid content in all groups, along with 20:5 and 22:5 fatty acids specifically for the HT_4 group. The increase in the PUFA content also led to a slight, but significant, increase in the average chain length.

Table 3. *Liver antioxidants in liver from control, cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated rats*

Parameter	C	CE	HT_3	HT_4
GPX	63.8±1.9	67.7±1.5	76.6±1.1*,†	54.1±1.1*,†,‡
GR	11.0±0.3	13.0±0.1*	16.9±0.3*,†	11.6±0.3†,‡
Vit E	27.9±1.2	34.1±0.9*	42.4±1.3*,†	33.4±1.5*,‡
CoQ9	111.6±2.5	132.0±2.7*	155.7±6.5*,†	114.0±3.0†,‡
CoQ10	13.3±0.6	16.0±1.0	20.3±1.8*,†	15.8±0.4‡
GSH	5.01±0.18	2.60±0.03*	2.41±0.10*	2.71±0.08*

Glutathione peroxidase activity (GPX) is expressed in μmol NADPH min^{-1} g^{-1} tissue. Glutathione reductase (GR) is expressed in μmol NADPH min^{-1} g^{-1} tissue. Vitamin E (Vit E), coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) contents are expressed in nmol g^{-1} tissue. Reduced glutathione (GSH) is expressed in μmol g^{-1} tissue.

Values are means ±s.e.m. For each value eight rats were used.

*Significant ($P<0.05$) *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

Response to oxidative stress

The luminescence response to changes of concentration of the homogenates (Fig. 5) has previously been described by the equation: $E=a\cdot C/\exp(b\cdot C)$ (Di Meo et al., 1996; Venditti et al., 1999). The *a* value depends on the concentration of substances, such as cytochromes, able to react with H_2O_2 to produce $^{\bullet}OH$ radicals that induce the luminescent reaction. Conversely, the *b* value depends on the concentration of substances, particularly water-soluble ones, able to prevent the formation

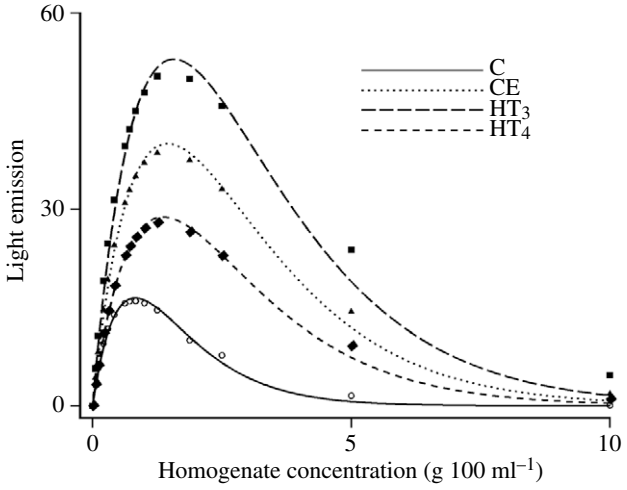

Fig. 5. Effect of cold exposure and hormonal [triodothyronine (T_3) or thyroxine (FT_4)] treatment on liver response to oxidative challenge *in vitro*. Tissue susceptibility to stress was evaluated by determining the variations, with concentration of homogenates, of light emission from a luminescent reaction. Emission values are given a percentages of an arbitrary standard (44 ng ml^{-1} peroxidase). The curves were computed from experimental data using equation $E=a\cdot C/\exp(b\cdot C)$. Preparations from control (C; solid line), 10-day cold exposed (CE; dotted line), T_3-treated (HT_3; broken line), and T_4-treated (HT_4; broken line) rats.

Table 4. *Fatty acyl composition and related indices of liver total lipids in control, cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated and rats*

Parameter	C	CE	HT_3	HT_4
14:0	0.69±0.008	0.53±0.02*	0.46±0.03*	0.52±0.03*
16:0	24.73±0.31	21.57±0.31*	19.47±0.41*,†	21.91±0.44*,‡
16:1n-7	2.89±0.16	1.74±0.10*	1.92±0.21*	2.12±0.14
18:0	16.58±0.44	21.69±0.35*	21.56±0.61*	20.24±0.38*
18:1n-9	11.81±0.27	8.75±0.29*	10.63±0.61†	9.14±0.27*,‡
18:2n-6	17.67±0.32	17.27±0.29	16.20±0.32*	16.81±0.35
18:3n-3	0.30±0.05	0.30±0.58	0.16±0.02*,†	0.23±0.01
20:3n-6	0.29±0.05	0.25±0.03	0.13±0.02*,†	0.24±0.03‡
20:4n-6	16.38±0.24	19.23±0.14*	21.77±0.38*,†	18.78±0.48*,‡
20:5n-3	0.49±0.01	0.48±0.03	0.33±0.08*	1.18±0.04*,†,‡
22:4n-6	0.37±0.04	0.37±0.02	0.47±0.02	0.40±0.03‡
22:5n-6	0.19±0.02	0.24±0.01	0.35±0.02*,†	0.26±0.02‡
22:5n-3	0.61±0.05	0.62±0.04	0.21±0.01*,†	1.14±0.11*,†,‡
22:6n-3	6.92±0.22	6.60±0.19	6.25±0.28	7.17±0.26‡
ACL	18.08±0.005	18.23±0.007*	18.29±0.01*,†	18.25±0.01*
SFA	42.01±0.44	43.79±0.57	41.51±0.66†	42.68±0.35
UFA	57.98±0.44	56.20±0.57	58.48±0.66†	57.31±0.35
MUFA	14.70±0.32	10.55±0.33*	12.56±0.79*,†	11.26±0.33*
PUFA	43.27±0.24	45.70±0.34*	45.92±0.57*	46.04±0.58*
PUFAn-6	34.93±0.38	37.38±0.49*	38.95±0.62*	36.31±0.74‡
PUFAn-3	8.34±0.14	8.31±0.19	6.97±0.27*,†	9.73±0.39*,†,‡
DBI	167.01±0.15	173.04±0.77*	176.94±1.89*	178.20±1.37*,†
PI	149.54±0.83	159.82±0.61*	164.59±2.2*,†	166.76±1.68*,†

Fatty acyl composition of lipids is expressed as mol %. For details on fatty acyl indices see the Materials and methods section.

Values are means ±s.e.m. For each value five C and CE rats, and six HT_3 and HT_4 rats were used.

*Significant *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

or interacting with •OH radicals, thus reducing the levels of light emission. Such levels, and particularly the emission maximum ($E_{max}=a/e\cdot b$), can be considered an index of the susceptibility of the preparations to oxidative challenge (Venditti et al., 1999). Thus, the curves in Fig. 5 indicate that tissue susceptibility to oxidants increases in hyperthyroid preparations reaching the highest and lowest values in T_3- and T_4-treated rats, respectively. These qualitative evaluations are confirmed by the E_{max} values (Table 5), the increases being the result of higher *a* values and lower *b* values. The differences in the *a* and *b* parameters between preparations from control- and hyperthyroid rats are consistent with the differences in COX activities and GSH levels, respectively.

Table 5. *Parameters characterising the response to oxidant of control, cold-exposed and 3,5,3′-triiodothyronine (T_3)- or thyroxine (T_4)-treated rat liver*

Parameter	C	CE	HT_3	HT_4
a	50.8±2.1	83.3±3.3*	83.7±5.5*	71.1±5.1*,†,‡
b	1.38±0.07	0.75±0.04*	0.64±0.08*	0.81±0.03*
E_{max}	13.5±0.7	40.8±1.3*	48.1±2.1*,†	32.7±1.1*,†,‡

For explanation of symbols see text. The relation between light emission and homogenate concentration of mitochondria is described by the equation: $E=a\cdot C/\exp(b\cdot C)$. $E_{max}=a/e\cdot b$.

Values are means ±s.e.m. For each value eight rats were used.

*Significant *vs* control (C) rats; †significant *vs* cold-exposed (CE) rats; ‡significant *vs* T_3-treated (HT_3) rats. The level of significance was chosen as $P<0.05$.

Discussion

We investigated the role of iodothyronine in rat liver response to cold, comparing it with those elicited by T_3 or T_4 treatments, which made serum FT_4 levels lower and higher, respectively, than those found in cold-exposed rats.

The changes in COX activities reveal enhanced liver oxidative capacity in CE rats, which can be attributed to high serum levels of T_3, because a similar increase was obtained by T_3 treatment. However, the changes found in both mitochondrial oxidative capacity and mitochondrial proteins in the liver suggest that the mechanisms underlying the tissue oxidative capacity increase is different in CE and HT_3 rats. In the CE group, this increase seems to be due to proliferation of mitochondria, which show little increase in their oxidative capacity, in agreement with the observation that the light mitochondrial fraction, characterized by low oxidative capacity, increases in chilled liver (Venditti et al., 2004c). Conversely, in the HT_3 group it is caused only by increase in mitochondrial

oxidative capacity. An intriguing possibility is that T_3 induces COX activation (and probably its synthesis) as well as mitochondrial proliferation, which, however, also requires T_4 levels higher than those present in HT_3 rats. This is supported by the finding that in HT_4 rats, in the presence of high FT_4 levels, moderate increases in FT_3 levels are associated with small increases in liver COX activity and mitochondrial proteins.

Unlike COX activity, liver state 3 respiration was differently affected by cold exposure and T_3-treatment. The moderate enhancement in tissue O_2 consumption produced by cold treatment could be explained by the small increase in mitochondrial COX activity causing a small increase in O_2 consumption by the respiratory chain, which is not compensated for by the increase in mitochondrial proteins. This is supported by the observation that tissue O_2 consumption, like mitochondrial COX activity, is not significantly different in HT_4 and CE rats.

The results concerning indicators of oxidative damage to lipid (HPs and MDAL) and proteins (carbonyls groups, GSA, and AASA) confirm the strict association between hypermetabolic state and oxidative stress occurring in hyperthyroid animals (Videla, 2000). However, they do not clarify what treatment causes the greatest oxidative damage in liver since the highest levels of hydroperoxides and protein-bound carbonyls were found in the HT_3 and CE groups, respectively, and their surrogate GC/MS markers in protein do not show similar behaviour in response to these treatments. In fact, carbonyl compound levels depend on the content of amino acids able to generate carbonyl groups in tissue proteins. Moreover, amino acids, such as lysine, can be subjected either to oxidative damage by ROS or to attachment by reactive carbonyl compounds formed by carbohydrate and fatty acid oxidation. The carbonyl-amine reactions can interfere with oxidative reactions in a measure dependent on extent of tissue peroxidative and glycooxidative processes. Therefore, the lower levels of protein-bound carbonyls in HT_3 than in CE rats should be consistent with the higher levels of hydroperoxides, CML and CEL found in T_3-treated animals. Nonetheless, concentration of protein adducts depends on both formation and breakdown, and in a severe hyperthyroid state protein degradation is so fast that there is a decrease in steady state CML and MDAL levels in rat liver (Guerrero et al., 1999). The lack of information on relative changes in protein degradation in the present experiments meant that it was not possible to quantify the interference between direct and indirect protein modifications. Thus, it is not clear what role, if any, T_4 plays in determining the differences in oxidative damage induced by cold or T_3 treatment. Moreover, the oxidative effects of the different treatments are mainly due to biochemical changes affecting free radical production and the antioxidant defence system.

In agreement with a previous report (Venditti et al., 2004b) we found that in CE rat mitochondria the rate of H_2O_2 generation increases only during state 4 respiration. Moreover, probably due to lower levels of autoxidizable electron carriers, this rate was significantly lower than in HT_3 rats. Despite this, mitochondrial ROS release strongly contributes to oxidative stress in cold liver because of the increase in mitochondrial proteins. The differences in free radical activity between HT_3 and CE rats do not seem to be due to T_4, in the light of the increased rate of mitochondrial H_2O_2 release during state 3 respiration see in T_4-treated rats.

Within the cell, in addition to the mitochondrial respiratory chain, there are other relevant sources of ROS, such as monoamine oxidase and microsomal monooxygenases. Oxidative deamination of biogenic amines catalysed by MAO, is a large source of H_2O_2 (Cadenas and Davies, 2000), the production of which, according to our results, should increase in CE and HT_4 rat livers because of their increased mitochondrial protein content.

The overall microsomal H_2O_2 production in liver from control rats (6.30 nmol min^{-1} g^{-1} liver) was 87.5% and 36.8% of the mitochondrial productions during state 4 respiration sustained by succinate and pyruvate/malate (7.2 and 17.1 nmol min^{-1} g^{-1} liver, respectively). Conversely, in all treatment groups, overall microsomal H_2O_2 production during pyruvate/malate-sustained state 4 respiration was about 55% of mitochondrial H_2O_2 production, suggesting an increased contribution by endoplasmic reticulum to tissue oxidative damage in the hyperthyroid state.

Treatments produced unbalanced and sometimes opposite changes in antioxidant enzyme activities and scavenger concentrations. Although the extent of some changes is directly related to serum T_3 levels and inversely to T_4 levels, this probably reflects a more important role for T_3 than for T_4 in oxidative protection. However, the GPX activity was lower in the HT_4 than in the control group, suggesting that it might be negatively regulated by T_4.

There was no clear relationship between lipid pattern and iodothyronine serum levels. Moreover, the degree of lipid unsaturation was not differentially increased by treatments, but the greatest susceptibility to peroxidative reactions was displayed by T_3- and T_4-treated rats.

Conversely, T_4 treatment induced the smallest decrease in liver capacity to oppose oxidative damage. The tissue susceptibilities to oxidants were in part related to the values of the parameter *a*, which depends on the tissue concentrations of substances, such as the hemoproteins, which are able to produce $^{\bullet}OH$ radicals (Halliwell and Gutteridge, 1990). Thus, the low *a* value found in HT_4 preparations was consistent with our observation that T_4 treatment slightly increased COX activity.

Overall, our results lead to the conclusion that the hyperthyroid state, by whatever treatment it is elicited, gives rise to increased liver oxidative capacity and oxidative damage, attributable to an action of T_3, which is the only iodothyronine for which circulating levels increase in all treatment groups. This idea is further supported by the observation that hepatic tissue, in which type I iodothyronine deiodinase is preferentially expressed, maintains thyroid hormone concentrations similar to those in plasma (Escobar-Morreale et al., 1997). However, it is apparent that there are differences in the size of the effects and underlying mechanisms, found in cold-exposed and T_3-treated rats. It is not clear whether this

depends on differences in T_4 serum levels. In fact, such a conclusion could be drawn, even in animals treated with deiodinase inhibitors, only if T_4 gave rise to changes opposite or greater than those produced by T_3. Unfortunately, this is difficult to find when measuring parameters such as oxidative damage extent, which depend on numerous factors, for which the relative contribution is not well defined. Thus, the only results suggesting differential effects of T_3 and T_4 concern parameters, such as GPX activity and mitochondrial protein content, more directly dependent on gene activity. Although the T_4-induced changes can supply an explanation of the different changes in the above parameters found in cold-exposed and T_3-treated rats, there is not enough evidence to indicate a role of T_4 in tissue response to cold exposure. However, it is a significant starting point for further experimental work, possibly performed on rats treated with T_4, which can be prevented from converting to T_3 by deiodinase inhibitors.

This work was supported by grants from the Spanish Ministry of Science and Technology (BFI2003-01287) and the Generalitat of Catalunya (2001SGR00311) and Italian Ministry of University and Scientific and Technological Research.

References

Barja de Quiroga, G., López-Torres, M., Perez-Campo, R., Abelenda, M., Nava, M. and Puerta, M. L. (1991). Effect of cold acclimation on GSH, antioxidant enzymes and lipid peroxidation in brown adipose tissue. *Biochem. J.* **277**, 289-292.

Barré, H., Bailly, L. and Rouanet, J. L. (1987). Increased oxidative capacity in skeletal muscles from acclimated ducklings: A comparison with rats. *Comp. Biochem. Physiol.* **88B**, 519-522.

Cadenas, E. and Davies, K. J. A. (2000). Mitochondrial free radical generation, oxidative stress and aging. *Free Radic. Biol. Med.* **29**, 222-230.

Cageao, L. F., Mignone, I. R., Ricci, C. R., Brignone, C. C., Brignone, J. A. and Zaninovich, A. A. (1992). Effects of thyroid hormones on mitochondrial oxygen consumption in brown adipose tissue and heart from cold-exposed hypothyroid rats. *Acta Endocrinol.* **127**, 72-75.

Carlberg, I. and Mannervik, B. (1985). Glutathione reductase. *Methods Enzymol.* **113**, 484-499.

Di Meo, S., Venditti, P. and De Leo, T. (1996). Tissue protection against oxidative stress. *Experientia* **52**, 786-794.

Escobar-Morreale, H. F., Obregon, M. J., Hernandez, A., Escobar del Rey, F. and Morreale de Escobar, G. (1997). Regulation of iodothyronine deiodinase activity as studied in thyroidectomized rats infused with thyroxine or triiodothyronine. *Endocrinology* **138**, 2559-2568.

Fernández, V. and Videla, L. A. (1993). Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles. *Free Radic. Res. Commun.* **18**, 329-335.

Flohé, L. and Günzler, W. A. (1984). Glutathione peroxidase. *Methods Enzymol.* **105**, 115-121.

Goglia, F., Liverini, G., De Leo, T. and Barletta, A. (1983). Thyroid state and mitochondrial population during cold exposure. *Pflügers Arch.* **396**, 49-53.

Gornall, A. G., Bardawill, C. J. and David, M. M. (1949). Determination of serum proteins by mean of the biuret reaction. *J. Biol. Chem.* **177**, 751-766.

Griffith, O. W. (1980). Determination of glutathione disulphide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**, 207-212.

Guernsey, D. L. and Stevens, E. D. (1977). The cell membrane sodium pump as a mechanism for increasing thermogenesis during cold acclimation in rats. *Science* **186**, 908-910.

Guerrero, A., Pamplona, R., Portero-Otin, M., Barja, G. and López-Torres, M. (1999). Effect of thyroid status on lipid composition and peroxidation in the mouse liver. *Free Radic. Biol. Med.* **26**, 73-80.

Halliwell, B. and Gutteridge, J. M. C. (1990). Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol.* **186**, 1-85.

Heath, R. L. and Tappel, A. L. (1976). A new sensitive assay for the measurement of hydroperoxides. *Anal. Biochem.* **76**, 184-191.

Himms-Hagen, J. (1983). Brown adipose tissue thermogenesis in obese animals. *Nutr. Rev.* **41**, 261-267.

Hyslop, P. A. and Sklar, L. A. (1984). A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. *Anal. Biochem.* **141**, 280-286.

Jansky, L. (1963). Body organ thermogenesis of the rat during exposure to cold and at maximal metabolic rate. *Fed. Proc.* **5**, 1297-1302.

Katewa, S. D. and Katyare, S. S. (2003). A semplified method for inorganic phosphate determination and its application for phosphate analysis in enzyme assays. *Anal. Biochem.* **323**, 180-187.

Kolosova, N. G., Kolpakov, A. R. and Panin, L. E. (1995). Tocopherol level and lipid peroxidation in Wistar rat tissues during adaptation to cold. *Vop. Med. Khim.* **41**, 16-19.

Lang, J. K., Gohil, K. and Packer, L. (1986). Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Anal. Biochem.* **157**, 106-116.

Nejad, I. F., Bollinger, J. A., Mitnich, M. and Reichlin, S. (1972). Importance of T_3 (triiodothyronine) secretion in altered states of thyroid function in the rat: cold exposure, subtotal thyroidectomy, and hypophysectomy. *Trans. Assoc. Am. Physicians* **85**, 295.

Pamplona, R., Portero-Otin, M., Riba, D., Ruiz, C., Prat, J., Bellmunt, M. J. and Barja, G. (1998). Mitochondrial membrane peroxidizability index is inversely related to maximum life span in mammals. *J. Lipid Res.* **9**, 1989-1994.

Pamplona, R., Dalfo, E., Ayala, V., Bellmunt, M. J., Prat, J., Ferrer, I. and Portero-Otin, M. (2005). Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. *J. Biol. Chem.* **280**, 21522-21530.

Reznick, A. Z. and Packer, L. (1994). Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol.* **233**, 357-363.

Simon, L. M. and Robin, E. D. (1971). Relationship of cytochrome oxidase activity to vertebrate total and organ oxygen consumption. *Int. J. Biochem.* **2**, 560-573.

Tnimov, M. Kh., Semeniuk, A. V., Voronina, N. P., Shishkina, L. N. and Maianskii, D. N. (1984). Effect of low temperature on lipid peroxidation in the lungs and on pulmonary macrophage activity in rats. *Biull. Eksp. Biol. Med.* **98**, 591-593.

Venditti, P., Balestrieri, M., Di Meo, S. and De Leo, T. (1997). Effect of thyroid state on lipid peroxidation, antioxidant defences, and susceptibility to oxidative stress in rat tissues. *J. Endocrinol.* **155**, 151-157.

Venditti, P., De Leo, T. and Di Meo, S. (1999). Determination of tissue susceptibility to oxidative stress by enhanced luminescence technique. *Methods Enzymol.* **300**, 245-252.

Venditti, P., De Rosa, R. and Di Meo, S. (2003a). Effect of thyroid state on H_2O_2 production by rat liver mitochondria. *Mol. Cell Endocrinol.* **205**, 185-192.

Venditti, P., Puca, A. and Di Meo, S. (2003b). Effect of thyroid state on H_2O_2 production by rat heart mitochondria: sites of production with Complex I and Complex II-linked substrates. *Horm. Metab. Res.* **35**, 55-61.

Venditti, P., Puca, A. and Di Meo, S. (2003c). Effect of thyroid state on rate and sites of H_2O_2 production in rat skeletal muscle mitochondria. *Arch. Biochem. Biophys.* **411**, 121-128.

Venditti, P., De Rosa, R., Portero-Otin, M., Pamplona, R. and Di Meo, S. (2004a). Cold-induced hyperthyroidism produces oxidative damage in rat tissues and increases susceptibility to oxidants. *Int. J. Biochem. Cell Biol.* **36**, 1319-1331.

Venditti, P., De Rosa, R. and Di Meo, S. (2004b). Effect of cold-induced hyperthyroidism on H_2O_2 production and susceptibility to stress conditions. *Free Radic. Biol. Med.* **36**, 348-358.

Venditti, P., De Rosa, R., Caldarone, G. and Di Meo, S. (2004c). Functional and biochemical characteristics of mitochondrial fractions from rat liver in cold-induced oxidative stress. *Cell. Mol. Life Sci.* **61**, 3104-3116.

Videla, L. A. (2000). Energy metabolism, thyroid calorigenesis, and oxidative stress: functional and cytotoxic consequences. *Redox Rep.* **5**, 265-275.