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Review

Thyroid hormone-induced oxidative stress

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Abstract. Hypermetabolic state in hyperthyroidism is associated with tissue oxidative injury. Available data indicate that hyperthyroid tissues exhibit an increased ROS and RNS production. The increased mitochondrial ROS generation is a side effect of the enhanced level of electron carriers, by which hyperthyroid tissues increase their metabolic capacity. Investigations of antioxidant defence system have returned controversial results. Moreover, other thyroid hormone-linked biochemical changes increase tissue susceptibility to oxidative challenge, which exacerbates the injury and dysfunction they suffer under stressful conditions. Mitochondria, as a primary target for oxidative stress, might account for hyperthyroidism-linked tissue dysfunction. This is consistent with the inverse relationship found between functional recovery of ischemic hyperthyroid hearts and mitochondrial oxidative damage and respiration impairment. However, thyroid hormone-activated mitochondrial mechanisms provide protection against excessive tissue dysfunction, including increased expression of uncoupling proteins, proteolytic enzymes and transcriptional coactivator PGC-1, and stimulate opening of permeability transition pores.

Key words. Thyroid hormones; oxidative stress; ROS production; antioxidant capacity; mitochondria.

Introduction

A major threat to homeostasis and therefore to the integrity of aerobic organisms arises from chemical species possessing one or more unpaired electrons, called free radicals. They can be generated *in vivo* as by-products of normal metabolic processes that are essential to the cell [1]. Aerobic organisms use oxygen to oxidise carbon- and hydrogen-rich substrates to obtain the energy essential for life. When organic molecules are oxidised, oxygen is reduced to form water by concerted four-electron transfer. However, oxygen can also undergo univalent reduction by one-electron transfer, which allows the formation of oxygen radicals and other reactive species (ROS), such as the superoxide anion radical ($^{\bullet}O_2^-$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). ROS can attack polyunsaturated fatty acids in the biomembrane (causing a chain of peroxidative reactions) proteins and enzymes (damaging functional properties), and nucleic acids (causing strand breaks and altered bases) [1, 2]. To neutralise the oxidative effects of ROS, the evolutionary survival process has provided aerobic organisms with a system of biochemical defences [3]. When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops [4]. This phenomenon has been related to many pathological conditions [5, 6], and it has also been suggested that some complications of hyperthyroidism are due to thyroid-hormone-induced oxidative stress in target tissues [7].

A primary intracellular target for oxidative stress, which might account for thyroid hormone-induced tissue damage, is represented by the mitochondria, the powerhouses of cellular life, which are also the motor of cell death.

Thus, after examining experimental evidence indicating that oxidative stress occurs in tissues from hyperthyroid animals, the present review focuses on the mechanisms by which excess of thyroid hormone could alter pro-oxidant-antioxidant balance characteristic of euthyroid tis-

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Table 1. Changes in indices of oxidative stress in hyperthyroid tissues.

	TBARS	HPs	Chemi-luminescence	MDAL	C=O	8-oxodG
Liver	↑(11–16) ↔(18) ↓(35)	↑(12, 14)	↑(11, 17)	↓(19)	↑(15)	↑(45) ↔(19)
Heart	↑(14, 18, 22–24) ↔(24, 34)	↑(14)				↔(44) ↓(32)
EDL	↔(18, 28)					
Soleus	↑(18, 25, 28)					
Gastrocnemious	↑(14, 31)	↑(14)				
White portion	↓(25)					
Hindlimb	↑(33)					↓(33)
Brain	↑(20, 21)		↑(20)			
MLN	↑(25)					
Thymus	↑(25)					
Spleen	↔(25)					
Kidney	↔(32)					
Testis	↔(27)	↓(27)			↑(27)	
BAT	↑(36) ↔(36)					
Blood	↑(37–43)	↑(43)				

TBARS, thiobarbituric acid reactive substances; HPs, hydroperoxides; MDAL, N^{ε}-(malondialdehyde)lysine; C=O, protein-bound carbonyls; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine. EDL, extensor digitorum longus; MLN, mesenteric lymph nodes; BAT, brown adipose tissue.

↑, ↓, and ↔ indicate hyperthyroid tissue levels increased, decreased and unchanged, respectively, in comparison with euthyroid controls.

sues, the relationship between oxidative stress and the deleterious effects of hyperthyroidism, and possible involvement of mitochondrial derangement.

Oxidative stress in hyperthyroid tissues

Oxidatively damaged lipids, proteins and DNA have been suggested as indices of oxidative stress in biological systems [8–10].

Lipid peroxidation

Lipid peroxidation has been frequently used as an index of oxidative stress in hyperthyroid tissues because polyunsatured fatty acids are especially susceptible to ROS attack and by-products of lipid peroxidation are easily measured. Hormonal effects on lipid peroxidation have been the subject of investigation in several laboratories. As showed in table 1, sometimes there are discrepancies among results, which seem to reflect a dependence of peroxidative processes on various factors, such as tissue, species, the iodothyronine used and treatment duration. On the other hand, it is not possible to exclude that some conflicting results depend on the differing accuracies of the methods used for lipid peroxidation determination. Thus, for example, the method for evaluating thiobarbituric acid reactive substances (TBARS) is inaccurate, and returns results which differ according to the assay conditions used [1].

In rat liver, T_3-induced hyperthyroidism was found to be associated with altered lipid peroxidation indices including elevated levels of TBARS [11–16] and hydroperoxides (HPs) [12, 14] in homogenates, and augmented spontaneous chemiluminescence in homogenates [11] and in *in situ* tissue [17]. Conversely, no change in TBARS was found in liver homogenate from rats made hyperthyroid by administration of T_4 to their drinking water over a 4-week period [18]. Interestingly, following the above treatment no significant increase was found in oxidative metabolism observed in liver from T_3-treated rats [11].

A decrease in liver content of N^{ε}-(malondialdehyde)lysine (MDAL), an adduct generated by reaction between lipid peroxidation-derived aldehyde, such as malondial-

dehyde (MDA) and lysine residue in proteins, was found in T_3-treated rats [19]. Although this might be viewed as an indication of decreased oxidative stress, the finding that protein degradation was the most relevant variable in determining the decrease in MDAL levels suggested increased oxidative stress in hyperthyroid liver [19].

Peroxidative effects elicited by thyroid hormones were found in the brain of newborn [20] and adult [21] rats. Such effects were also found in heart homogenates [14, 18, 22, 23] from young rats. However, increased lipid peroxidation in hearts from old (1.5 years) but not from young (8 weeks) hyperthyroid rats was also reported [24]. Thyroid hormone treatment was found to increase lipid peroxidation in lymphoid organs such as mesenteric lymph nodes and thymus, without major effects in the spleen [25], a thyroid hormone-unresponsive tissue [26]. Thus, no significant change (TBARS) or decrease (HPs) were observed in lipid peroxidation level in the testis from adult hyperthyroid rats [27], and the thyroid hormone-induced increase in lipid peroxidation was found to be confined to some skeletal muscles. In both rat [18, 25] and cat [28], such an increase was found in the soleus, a red muscle mainly composed of slow-twitch oxidative glycolitic fibres (type I). Conversely, no change was found in the extensor digitorum longus (EDL) [18, 28], a white muscle mainly composed of fast-twitch glycolitic fibres (Type IIb). These results are consistent with early observations that red, but not white muscles, are sensitive to thyroid hormones [29, 30]. Lipid peroxidation was also increased by thyroid hormone in rat gastrocnemious [14, 31], a mixed fibre muscle also containing fast-twitch oxidative glycolytic fibers (type IIa), but was decreased in the white portion of such a muscle [25].

On the other hand, it is surprising that in kidney from hyperthyroid rats the lipid peroxidation level does not change [32], although the tissue exhibits a calorigenic response to thyroid hormone similar to that elicited in liver [26].

Studies on the mouse showed lower susceptibility to thyroid hormone-induced lipid oxidative damage. Indeed, levels of lipid peroxidation were found to be increased in hindlimb muscles [33], unchanged in heart [34] and decreased in liver [35] from hyperthyroid mice. The results concerning liver were attributed by the authors to the animal species or long-term (4–5 weeks) treatment they used, because a laboratory study describing no increase in index of lipid peroxidation in hyperthyroid rat liver used the same long-term treatment [18]. Although this may be true, it is interesting that in both mouse and rat hyperthyroidism was induced by T_4, whose biological activity can differ from T_3 in some tissues. Indeed, recent studies have shown that T_4, but not T_3, increases lipid peroxidation in rat interscapular brown adipose tissue (BAT) [36].

It is well established that thyroid hormone also induces lipid oxidative damage in man. Clear evidence for this was provided by increased circulating levels of parameters related to peroxidative processes, such as TBARS [37–43] and HPs [43], in hyperthyroid patients.

Protein and DNA oxidative damage

There are not many data regarding the effect of the thyroid state on protein and DNA oxidation (Table 1). However, it does seem that, in addition to lipid peroxidation, hyperthyroidism promotes protein oxidation in rat liver [15] and testis [27], as evidenced by enhanced content of protein-bound carbonyls. While protein damage was already shown after 3 days of T_3 treatment, oxidative damage to DNA, estimated as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), was not found increased in liver [19] and heart [44] from rats made hyperthyroid by 10-day T_3 treatment. Conversely, it has been recently reported that longer-term (25 days) T_4 treatment is associated with increased 8-oxo-dG levels in rat liver [45]. Some explanations can be provided for the lack of 8-oxodG increase after short-term treatment. First, it is likely that the most of the H_2O_2 generated by various cellular sources is intercepted by cytosolic antioxidants before it reaches the nucleus, where the vast majority of genomic DNA is located. Second, nuclear DNA is extensively covered by proteins such as histones, which make it less susceptible to ROS attack [46]. Finally, 8-oxodG is rapidly repaired by a specific 8-oxoguanine DNA glycosylase/lyase [47], and increases in oxidative stress induce enhanced repair of DNA oxidative damage [48]. The repair activity of genomic DNA during thyroid hormone-induced oxidative stress seems to be particularly relevant in mouse skeletal muscles. Indeed, oxidative damage of mitochondrial DNA does not increase, and that of nuclear DNA decreases not only in heart [32] but also in hindlimb muscles from hyperthyroid mice [33].

Mechanisms of oxidative stress

Despite some contradictory reports, the aforementioned results provide strong evidence that thyroid hormones induce oxidative stress in target tissues. Oxidative stress results from a disturbance of the normal cell balance between production of ROS and the capacity to neutralise their action. Thus, in order to understand why oxidative stress develops in some physio-pathological conditions, it is worthwhile examining the changes in tissue capacity to generate and neutralise free radicals.

Changes in free-radical production

In aerobic cells O_2 is mainly consumed through its four-electron reduction to water by cytochrome c oxidase. This reaction occurs without release of any intermediate in the

O_2 reduction. However, despite the efficiency of the mitochondrial electron transport system, the nature of the alternating one-electron oxidation-reduction reactions it catalyses predisposes electron carriers to side reactions, in which an electron is transferred to O_2 directly, instead of to the next electron carrier in the chain, generating $O_2^{\bullet-}$ [49]. This radical is then converted by spontaneous or catalysed dismutation into hydrogen peroxide (H_2O_2) [50], which can be turned into highly reactive hydroxyl radical ($^{\bullet}OH$). Numerous oxidases in the cytosol, endoplasmic reticulum and outer mitochondrial membrane also contribute to O_2 consumption and lead to $O_2^{\bullet-}$ and H_2O_2 generation [5].

In most (if not all) mammalian cells, another free radical, containing nitrogen and named nitric oxide ($NO^{\bullet}$), is synthesised from L-arginine by NO synthases (NOS) in an NADPH-requiring reaction [51]. At physiological concentration $NO^{\bullet}$ is relatively unreactive, and its physiological activity is mostly mediated by binding to Fe^{2+} in the heme of soluble guanylate cyclase, causing enzyme activation and cyclic GMP (cGMP) production [52]. However, $NO^{\bullet}$ may be converted to a number of more reactive derivatives, known collectively as reactive nitrogen species (RNS). Thus, reacting with superoxide, $NO^{\bullet}$ produces peroxynitrite ($ONOO^-$), a powerful oxidant, able to damage many biological molecules and both decompose releasing small amounts of hydroxyl radicals [53].

Changes in ROS and RNS production

The literature concerning the effects of thyroid hormone on ROS and RNS production is poor, but there is convincing evidence for increased radical production in the hyperthyroid state.

RNS production

Fernández et al. [54] found that T_3 administration for 3 consecutive days leads to a progressive and significant enhancement in rat liver NOS activity, an effect which is reversed upon hormone withdrawal. Hepatic synthesis of $NO^{\bullet}$ occurs in both hepatocytes [55] and Kupffer cells [56], and the enhancement in liver NOS, achieved by T_3 treatment, seems to be exerted at least in both cell types. It was also suggested that thyroid hormone-induced $O_2^{\bullet-}$ and $NO^{\bullet}$ generation likely leads to peroxynitrite ($ONOO^-$) formation that may contribute to the enhanced pro-oxidant condition elicited by hyperthyroidism in the liver [52]. This effect occurs notwithstanding hyperthyroidism is also associated with decreased mitochondrial NOS activity [57].

Subsequent studies have shown that thyroid hormone induces upregulation of NOS gene expression in rat hypothalamus [58] and $NO^{\bullet}$ overproduction by human vascular endothelium [59] and rat phagocytic cells [60]. Recently, indirect evidence has been provided that $NO^{\bullet}$ production also increases in hyperthyroid heart [61, 62].

ROS production

Only one paper deals with microsomal ROS production in hyperthyroidism. It reported an increased rate of NADPH-supported generation of superoxide radical by microsomal fractions from rat liver after 2 (30%) to 7 (67%) days of treatment of euthyroid rats with T_3 [11]. This was in agreement with concomitant elevation in microsomal NADPH oxidase activity, which has been shown to be associated with $O_2^{\bullet-}$ production [63].

In contrast, several papers showed that hyperthyroidism induces an increase in mitochondrial ROS generation. Swaroop and Ramasarma [64] found that rat exposure to heat decreases H_2O_2 release by intact mitochondria from liver, but not from kidney or heart. With all the substrates (succinate, glycerol-1-phosphate and choline) decreases by about 50% in the first 2–3 days, and further decreases after 5–6 days of exposure were obtained. These decreases paralleled the decreases in serum T_4 levels and were attributed to the hypothyroid state of the animals. This was confirmed by the large decrease in H_2O_2 generation found under two other hypothyroid conditions produced by propylthiouracil (PTU) or thyroidectomy. Furthermore, treatment of heat-exposed or thyroidectomized rats with a single dose of T_4 12 h before killing stimulated H_2O_2 generation. Stimulation of H_2O_2 generation was also obtained by treatment of euthyroid rats with single dose or a 3-day dose of T_4 [64].

Unfortunately, mitochondral H_2O_2 generation in kidney and heart of T_4-treated rats was not determined, so at present it is not known whether T_4 administration also produces increased mitochondrial H_2O_2 release in other tissues.

Subsequently, it was found that liver submitochondrial particles from T_3-treated rats exhibited enhanced rate of succinate or NADH-supported $O_2^{\bullet-}$ production, and succinate-supported H_2O_2 production in the absence and in the presence of antimycin-A (AA), a mitochondrial respiration inhibitor [65]. Interestingly, it was also found that thyroid hormone, which increases the number of liver peroxisomes [66], increases the rate of urate-supported AA-insensitive H_2O_2 generation by preparations of submitochondrial particles [11], suggesting a peroxisome role in hyperthyroid state-linked enhancement in liver ROS production.

Recently, the rate of H_2O_2 release by liver [67], skeletal muscle (gastrocnemious) [68] and heart [69] intact mitochondria from hypothyroid rats made hyperthyroid by 10-day T_3 administration has been evaluated, using complex I and complex II-linked substrates (succinate and pyruvate plus malate, respectively). Thus, it has been found that, with all substrates, the rate of H_2O_2 release by liver and heart mitochondria increases in both basal (state 4) and ADP-stimulated (state 3) respiration, while that by muscle mitochondria increases only during basal respiration.

Table 2. Effect of hyperthyroidism on tissue antioxidants.

	Blood	Heart	Liver	EDL	Soleus	Gastro-cnemious	Hindlimb	Brain	BAT
Mn-SOD		↑(18, 24, 105, 106) ↔(107)			↑(18, 25, 106)	↑(25)			↑(36) ↔(36)
Cu, Zn-SOD	↑(39, 40)	↔(18, 24, 107) ↓(22, 105, 106)	↓(18, 108)	↔(18)	↔(18) ↓(106)	↑(25, 31)		↑(20)	↔(36) ↓(36)
Total SOD		↑(24, 107)	↓(17)						
CAT		↔(24, 105, 107) ↓(18, 106)	↓(17, 18)	↔(18)	↑(25) ↓(18, 106)	↑(25)		↑(20)	↓(36)
GPX	↓(38, 40)	↑(22) ↔(14, 24) ↓(18, 24, 106, 107)	↑(108) ↔(14, 109) ↓(18)	↓(18)	↓(18, 25, 106)	↑(14) ↓(25, 31)		↑(20)	
GR	↓(39)	↔(14)	↑(108) ↔(14, 109)			↔(14)			
Vitamin E	↓(38, 42) ↔(106)	↑(14, 24, 107)	↔(12, 14)		↔(106)	↔(14)			
CoQ9	↔(107, 110) ↓(110)	↔(110) ↓(107)	↑(110) ↔(110)						
CoQ10	↓(42)	↔(107)							
GSH	↑(39, 40, 108) ↓(41)	↔(34)	↔(35) ↓(12, 16, 17, 108, 109, 111)			↓(31)	↔(33)		
CA		↓(14)	↓(12, 14)			↔(14)			

EDL, extensor digitorum longus; BAT, Brown adipose tissue.
↑, increased; ↓, decreased; ↔, unchanged.

The results concerning heart mitochondria disagree with those obtained by López-Torres et al. [44], which reported that hyperthyroidism does not modify H_2O_2 release by heart mitochondria during pyruvate/malate-supported state 4 and state 3 respiration. The discrepant results could be explained by different procedure used for the preparation of the mitochondria. The former protocol [69] provided incubation of tissue fragments with nagarse, removal of nuclei and cell debris at 500 g, and sedimentation of mitochondria at 3000 g. Conversely, the latter protocol [44] provided no incubation and centrifugation at 700 and 8000 g, respectively. It is therefore likely that the preparations contained different amounts of microsomal contamination and heavy mitochondria characterised by a high capacity to produce ROS [70]. It is worth noting that the results of López-Torres [44] suggested that even though there was no increase in H_2O_2 release per mg of mitochondrial protein, the release per gram of tissue was higher in hyperthyroid heart because of the increase of total mitochondrial proteins. Thus, both reports led to the same conclusion that H_2O_2 release contributes to oxidative stress, which characterises hyperthyroid heart.

Hydrogen peroxide produced within intact mitochondria is partially removed by H_2O_2-metabolizing enzymes located in the matrix and hemoproteins located in the inner membrane [71]. Therefore, determination of the rate of hydroperoxide release does not allow one to deduce anything about the rate of production, even though it is obvious that respiratory chain must produce more H_2O_2 than that released by intact organelles. A method to measure the capacity of mitochondria to remove H_2O_2 has been pointed out, but its failure to express it as rate of H_2O_2 removal does not enable calculation of the hydroperoxide production rate [71]. However, the observation that the capacity of H_2O_2 removal of liver [67], muscle [68] and heart [69] mitochondria increases in the hyperthyroid state has demonstrated that thyroid hormone accelerates H_2O_2 production during basal and ADP-stimulated respiration in such tissues.

Mitochondrial sites of ROS production

Within mitochondria, H_2O_2 rises by dismutation of $O_2^{\bullet-}$ radical catalysed by manganese-dependent superoxide dismutase (MnSOD) [72]. In heart mitochondria $O_2^{\bullet-}$ generation seems to occur at an Fe-S centre of Complex I [50] and at a segment between NADH dehydrogenase and ubiquinone/cytochrome b of Complex III [73]. The early view that Complex III is quantitatively more active than Complex I in ROS generation [50, 74] has been questioned by Hansford et al. [75]. Furthermore, tissue- and species-linked differences in the sites of $O_2^{\bullet-}$ generation have been found [76], so it is conceivable that the contribution of ROS generators in mitochondria of the various tissues can be differently modified by an altered thyroid state. Information on this matter has been obtained by analysing the effects of respiratory inhibitors and substrates on H_2O_2 production.

The results of such analysis suggest that: (i) in all thyroid states pyruvate/malate is a more effective substrate than succinate for H_2O_2 generation by liver [67], muscle [68] and heart [69] mitochondria; (ii) within heart mitochondria, the major locus for $O_2^{\bullet-}$ production is Complex III in both euthyroid and hyperthyroid states [44, 69]; (iii) within liver mitochondria, Complex I is the major site of ROS generation in all thyroid states [68]. About muscle mitochondria, although it is apparent that, in both euthyroid and hyperthyroid states, $O_2^{\bullet-}$ production occurs at both Complex I and Complex III, it is not clear where the main production site is located [68].

Biochemical changes determining increased ROS production

Available results indicate that thyroid hormone treatment increases H_2O_2 generation from rat tissue mitochondria, but it is not clear why this happens. Although the idea is widely shared that in healthy tissues the majority of ROS are produced by mitochondria, few studies have been performed on the physiological factors which regulate the rate of mitochondrial free-radical production. It has been frequently assumed that such a rate increases in proportion to the rate of oxygen consumption. This idea seemed to be supported by the observation that the extent of oxidative damage found following thyroid hormone treatment [7], cold exposure [77], exercise [77] and caloric restriction [78] was related to the organism's metabolic state. In effect, the hyperthyroid functional state induced in mammals by cold exposure, like experimental hyperthyroidism, leads to enhancement of their basal metabolic rate [79], which reflects increases in the cellular respiration of target tissues as BAT [77], liver, and cardiac and skeletal muscles [80]. However, a recent report has shown that during both pyruvate/malate and succinate-supported state 4 respiration, H_2O_2 generation by liver mitochondria from cold-exposed rats is accelerated without any change in the O_2 consumption rate [81]. Moreover, despite some contradictory reports, no differences in whole-body metabolic rate [82] and mitochondrial O_2 consumption [83] between caloric restricted and ad libitum-fed rats have been reported, while the rate of mitochondrial H_2O_2 release seems to be reduced by caloric restriction [84, 85].

On the other hand, the dependence of ROS production on O_2 consumption should require that the percentage of total electron flow escaping from the respiratory chain to reduce O_2 to oxygen radical (the mitochondrial free radical leak) is not substantially modified during the transition from basal to ADP-stimulated respiration. There are at least two reasons to rebut such an idea. First, both cardiac and skeletal muscle have antioxidant concentrations more than one order of magnitude lower than those of

other highly aerobic tissues such as liver [86]. Therefore, if ROS production increases in proportion to oxygen consumption, the muscles will undergo massive oxidative damage during an acute bout of exercise. Second, ROS production decreases when ADP phosphorylation increases (state 3). Early studies using succinate, a complex-II-linked substrate, found that mitochondrial ROS production halts after the addition of sufficient ADP to cause the state 4-to-state 3 transition [73, 87–89]. This finding generated the widespread notion that mitochondria only produce free radicals in the resting state 4 condition. More recent studies found significant H_2O_2 release in the presence of ADP in succinate-supplemented mitochondria from several rat tissues, including BAT, spleen, thymus [90], liver [67, 90], heart [69, 91] and skeletal muscle [68, 92]. However, the above reports showed decreased ROS production during ADP-stimulated respiration, indicating that free-radical leak in the various tissues strongly decreases in state 3. On the other hand, this agrees with the observation that the rate of the mitochondrial ROS generation is related to the degree of reduction of the oxidisable electron carriers [73, 74, 87], which decreases strongly during the state 4-to-state 3 transition together with the increased electron flow through the respiratory chain [93]. In this light, enhancement in ROS production found in mitochondria from hyperthyroid tissues could be due to an increase in content and reduction degree of the oxidisable electron carriers.

It is worth noting that the long-term effects of thyroid hormone on mitochondrial respiration are obtained by increasing the content of electron carriers, such as cytochromes [94, 95] and ubiquinone [96, 97], and their percent reduction [95, 98]. Moreover, measurements of the H_2O_2 generation rate in the presence of antimycin A indicate that even mitochondrial concentration of oxidisable electron carriers increases in hyperthyroid tissues [67, 69]. These results strongly support the idea that an increase in mitochondrial ROS generation, underlying cellular oxidative damage, is a side effect of the thyroid hormone-induced biochemical changes by which animal tissues increase their metabolic capacity.

Kupffer cell involvement in thyroid hormone-induced oxidative stress

Although the most of the increased ROS and RNS production observed in liver of hyperthyroid rats occurs at the parenchymal cell level, several reports indicate that a substantial contribution is supplied by Kupffer cells. Indeed, a significant portion of the T_3-induced increase in liver lipid peroxidation and NOS activity is abolished by gadolinium chloride ($GdCl_3$) inactivation of Kupffer cells [54, 99].

It has also been shown that T_3 administration to rats produces transient activation of hepatic nuclear factor-*k*B [100], a redox-sensitive factor responsible for the transcriptional activation of cytokine-encoding genes. It appears that such an activation involves a role for T_3-induced free-radical activity on Kupffer cells because it is decreased by *in vivo* treatment with α-tocopherol and N-acetylcysteine and abolished by GdCl3 treatment. Moreover, livers from hyperthyroid rats with enhanced NF-κB DNA binding activity show induced messenger RNA (mRNA) expression of the NF-κB-responsive genes for tumor necrosis factor-α (TNF-α) [100], a cytokine known to stimulate ROS generation [101]. In turn, the early increase in TNF-α production may contribute to the late NF-κB activation considering that TNF-α promotes the phosphorylation of both the inhibitory IκB proteins, allowing their degradation [102], and NF-κB p65 subunit, which stimulates the transcriptional activity of NF-κB [103]. However, the transient increment in the circulating levels of TNF-α [100] suggests that the establishment of a dangerous positive feedback loop is avoided. This can be due to the late effect of T_3 on the expression of the NF-κB responsive gene for interleukin (IL)-10 [100], which is able to preserve expression of the inhibitory IκB protein [104].

Changes in the antioxidant defence system

Substances that neutralise the potential ill effects of free radicals are generally grouped in the so-called antioxidant defence system. Such a system includes both low molecular weight free-radical scavengers and a complex enzyme array involved in scavenging free radicals, terminating chain reactions, and removing or repairing damaged cell constituents. To provide maximum protection, these substances are strategically compartmentalised in subcellular organelles within the cell and act in concert.

In examining antioxidant changes found in hyperthyroid tissues, it needs to be underscored that although thyroid hormone can directly control levels of enzymes with antioxidant activity or regulate scavenger content, antioxidant depletion could not be the cause, but the consequence of the oxidative stress.

The effects of thyroid hormone on antioxidant status have been extensively investigated in rat tissues, while a few data concerning other species are available.

Antioxidant enzymes

As shown by the results reported in table 2, there is a tissue-linked variability in the effects of hyperthyroidism on antioxidant enzyme activities. Moreover, there are discrepancies in the changes observed in the same tissue, which sometimes seem to depend on animal age and the iodothyronine used for treatment.

In the rat, BAT Mn-SOD activity was found to increase after T_3, but not T_4, treatment [36]. However, cardiac activity was reported to both increase [18, 24, 105, 106] and remain unchanged [22, 107], even though in all cases hyper-

thyroidism was elicited by long-term treatment with T_4. Mn-SOD was also found to increase in the soleus and white portion of gastrocnemious muscle from rats made hyperthyroid by combined T_3 and T_4 administration [25] and in soleus [18, 106] and gastrocnemious [31] from T_4-treated rats.

Different effects of T_3 and T_4 were also shown by analysing the BAT activity of copper, zinc-dependent superoxide dismutase (Cu,Zn-SOD), which was decreased by T_3 and not modified by T_4 [36]. Moreover, Cu,Zn-SOD activity increased in gastrocnemious [31] and in its white portion [25], remained unchanged in EDL [18], in agreement with insensitivity of such muscle to thyroid hormone, whereas it was reported to both decrease [105, 106] and remain unchanged [18, 24, 107] in cardiac muscle, despite the same prolonged treatment with T_4. Total SOD was found to decrease in liver [17] and increase in heart from young [24, 107] but not from old [24] hyperthyroid rats.

For catalase (CAT) activity, together with the expected lack of change in EDL [18], an increase in the white portion of gastrocnemious [25] and both decrease [18, 106] and increase [25] were found in soleus from hyperthyroid rats. Decreases in CAT activities were found in BAT after T_3 or T_4 treatment [36] and in liver [18, 108], whereas lack of change [24, 105, 107] and decrease [18, 106] were found in heart.

The relationship between hyperthyroidism and glutathione peroxidase (GPX) activity also appears not well defined. Indeed, it was reported that cardiac activity decreased after long-term T_4 treatment of both young [18, 106, 107] and old [24] rats, increased [22] and remained unchanged [24] after long-term T_4 treatment of young rats, and remained unchanged after short-term T_3 treatment of young rats [14]. Liver GPX activity was found to decrease after T_4 treatment [18], but both increased [108] and remain unchanged [14, 109] after T_3 treatment. Moreover, it was found that T_3 treatment increased GPX activity in gastrocnemious [14], while T_4 and T_3+T_4 treatments decreased such activity in gastrocnemious [31] and in its white portion [25], respectively. T_4 administration also decreased GPX activity in both thyroid hormone responsive (soleus) [18, 106] and unresponsive (EDL) [18] muscles. Enzyme activity was found increased in brain from hyperthyroid newborn rats [20].

The changes induced by T_3 treatment in both liver [14, 108, 109] and heart [14], but not in muscle glutathione reductase (GR) activities shown in the various laboratories were consistent with those found for GPX activities.

It is interesting that in brain of newborn hyperthyroid rats the activities of antioxidant enzymes (Cu,Zn,-SOD, CAT and GPX) exhibited compensatory increase that did not prevent oxidative stress [20].

Finally, it was reported that Cu,Zn-SOD activity increased [39, 40], while GPX [38, 40] and GR [39] activities decreased in the blood of hyperthyroid patients.

Low molecular weight scavengers

The results of studies dealing with low molecular weight scavengers (table 2) show that, as expected, hyperthyroidism in man is associated with reduced circulating levels of the liposoluble antioxidants α-tocopherol [38, 42] and coenzyme Q10 [42]. Circulating levels of the water-soluble antioxidant glutathione (GSH) were found to both increase [39, 40] and decrease [41] in hyperthyroid patients. Lack of changes in GSH content was found in hindlimb muscles [33], heart [34] and liver [35] from hyperthyroid mice. Because the decrease in GSH levels, like the enhancement in lipid peroxidation, is considered an index of oxidative stress, it is not surprising that in the hyperthyroid state GSH levels decrease in human blood and remain unchanged in mouse liver and heart. Conversely, there is no straightforward explanation for the increase and the lack of changes in GSH levels found in hyperthyroid human blood and mouse muscle, respectively.

The changes in the levels of the liposoluble scavengers α-tocopherol and coenzyme Q induced by thyroid hormone in various rat tissues were found to be unbalanced and often opposite. Vitamin E levels in skeletal muscles [14, 106] and liver [12, 14] remained unchanged. Similar results were obtained for heart after long-term T_4 treatment [106]. However, other authors found increased heart vitamin levels not only after T_3 treatment of young rats [14], but even after long-term T_4 treatment of young [24, 107] and old [24] rats.

CoQ9 levels were determined on rats made hyperthyroid by T_4 treatment. Serum levels were found unchanged in mild [107, 110] and decreased in severe hyperthyroidism [110]; liver levels were found increased in mild [110] and unchanged in severe hyperthyroidism [110]; heart levels were found unchanged in severe hyperthyroidism [110], and both decreased [107] and unchanged [110] in mild hyperthyroidism obtained by 30- and 21-day treatments, respectively. After the former treatment heart levels of CoQ10 were found unchanged [107].

The levels of water-soluble antioxidant glutathione (GSH) were found to decrease in liver [12, 16, 17, 108, 109, 111] and gastrocnemious muscle [31], and increase in blood [108] from hyperthyroid rats.

The liver depletion of GSH is of great interest because this tripeptide is involved in many metabolic processes, including the prevention of oxidation of thiol groups on proteins that are essential for the stability and function of such macromolecules. Under normal steady-state conditions, the GSH concentration in liver is maintained by continuous synthesis and use, the latter being carried out by processes such as oxidation to GSSG, conjugation reactions, enzymatic breakdown initiated by γ-glutamyltransferase, and transport of GSH across canalicular and sinusoidal membranes [112].

In their investigation of mechanisms of the T_3 effects Fernández et al. [109] suggested that, even though GPX-GR

couple activity was not modified, the high rates of GSH oxidation were adequately balanced by the GSSG reduction system and the decreased activity of GSH transferases (GST), as GSSG accumulation in the tissues did not occur [17, 108]. The γ-glutamyl-transferase activity was found to exhibit a progressive and drastic enhancement under the influence of T_3 [109]. Moreover, T_3 treatment did not modify the canalicular rate of GSH efflux in the rat, whereas the sinusoidal component was drastically enhanced. The latter effect of hyperthyroidism was found in parallel with an enhancement in sinusoidal lactate dehydrogenase and protein release, suggesting that loss of GSH might be related to permeabilisation of the hepatocyte plasma membrane [109]. Thus, the increases in liver sinusoidal GSH efflux and capacity to degrade the tripeptide appear to be major mechanisms leading to GSH depletion in hyperthyroid liver. As increased GSH use is not balanced by an increase in GSH synthesis, a lower steady-state level of GSH is reached in the liver.

Apart from the discrepancies in the results, which are not explained by protocol differences, on the whole the thyroid hormone-induced changes in antioxidants in the various tissues does not seem to offer a coherent pattern. On the other hand, even with more consistent data, clearly measurements of selected antioxidants provide limited information regarding the total antioxidant status of the tissue. Indeed, this approach does not permit a complete appraisal of the synergistic cooperation of the various antioxidant systems. Therefore, the question remains: How is antioxidant defence system effectiveness modified in the hyperthyroid state? An appropriate approach to resolve this question should be to challenge tissues with specific free radicals *in vitro* to determine overall antioxidant capacity. Studies performing such experiments have shown that whole antioxidant capacity (CA) undergoes significant decrease in liver [12, 14] and heart [14], but not in gastrocnemious muscle [14] from hyperthyroid rats.

Tissue susceptibility to oxidants

The above results indicate that changes in the individual components of the tissue antioxidant defence system strongly reduce the global efficacy of such a system in hepatic and cardiac tissue, but not in gastrocnemious muscle from hyperthyroid rats. However, this does not provide information on sensitivity of the hyperthyroid tissues to oxidative processes. Indeed, other thyroid state-linked changes in biochemical characteristics of the tissues can modify their susceptibility to oxidants and the extent of the oxidative damage they suffer following oxidative challenge.

Lipid peroxidation is initiated after hydrogen abstraction from polyunsaturated fatty acids by $^{\bullet}OH$ radicals, and it is known that susceptibility of lipids to peroxidative processes increases as a function of number of the double bonds of their fatty acids.

Thyroid hormone ability to influence lipid composition in rat tissues is well known [113], so it is expected that lipid peroxidation can be modified in hyperthyroid tissues subjected to oxidative attack. In fact, it was found that the *in vitro* induction of TBARS by tert-butyl hydroperoxide, H_2O_2 or $FeSO_4$/ascorbic acid in brain from hyperthyroid rats was very high when compared with euthyroid rats [21]. Conversely, no difference was found between euthyroid and hyperthyroid preparations from adult rat testis [27]. Using $FeSO_4$/ascorbic acid, it was found that sensitivity to *in vitro* lipid peroxidation was increased in skeletal [33] and cardiac [34] muscles, and decreased in liver [35] from T_4-treated mice. Sensitivity of mouse liver to *in vitro* lipid peroxidation, as well as endogenous lipid peroxidation levels, correlated well with the results of the fatty acid composition analyses. Indeed, highly unsaturated 20:4n-6 and 20:3n-3, unsaturation and peroxidizability indexes as well as the double-bond index decreased in T_4-treated mice [35].

The content of high and low molecular weight Fe^{2+} complexes, which promote the conversion of H_2O_2 into $^{\bullet}OH$ radicals via the Fenton reaction [6, 71, 86], can also affect tissue susceptibility to ROS-induced damage. The extent of oxidative changes occurring under conditions leading to increased *in vivo* ROS production was evaluated by measuring the levels of light emission resulting from *in vitro* exposure to H_2O_2 of biological preparations. The relationship between light emission and homogenate concentration was supplied by the equation: $E = a \cdot C / \exp(b \cdot C)$, in which the parameters a and b were dependent on the concentrations of substances able to induce and inhibit, respectively, the H_2O_2-induced luminescent reaction [114]. Using this technique, it was established that susceptibility to oxidative challenge is higher in hyperthyroid than in euthyroid tissues, including liver [12, 14], heart [14] and muscle [14], because of higher *a* values and lower *b* values. On the whole, these results are consistent with the increase in cytochrome content [115] and the decrease in antioxidant capacity [14] found in hyperthyroid tissues.

Oxidative stress-linked dysfunction of hyperthyroid tissues

Thyroid hormones, of which T_3 is the major active form, exert a multitude of physiological effects affecting growth, development and metabolism of vertebrates [116], so that they can be considered major regulators of their homeostasis. On the other hand, elevated circulating levels of thyroid hormones are associated with modifications in the whole organism (weight loss and increased metabolism and temperature) and in several body regions. In-

deed, low plasma lipid levels, tachycardia, atrial arrhythmias, heart failure, muscle weakness and wasting are commonly found in hyperthyroid animals. Plasma membrane [117], endoplasmic reticulum [118] and mitochondria [119] have been considered as potential cellular sites of action of thyroid hormone. However, it is now generally accepted that most of the actions of thyroid hormone results from influences on trascription of T_3-responsive genes, which are mediated through nuclear thyroid hormone receptors [120]. It is worth noting that the idea that oxidative stress underlies dysfunctions produced by hyperthyroidism is not in contradiction with mediation of T_3 action through nuclear events. Indeed, it is conceivable that some of the biochemical changes favouring the establishment of the oxidative stress (increase in mitochondrial levels of electron carriers, NOS activity and the unsaturation degree of lipids) are due to stimulation of the expression of specific genes initiated through T_3 binding to nuclear receptors. Thyroid hormone induces upregulation of NOS gene expression in rat hypothalamus [58], and it is conceivable that this also happens in other tissues in which T_3-induced $NO^{\bullet}$ overproduction has been shown [54, 59, 62]. However, direct evidence for a relationship between the ill effects of hyperthyroidism and oxidative stress remains poor.

Thyrotoxic myopathy

Thyrotoxic myopathy is a well-known example of tissue damage due to the action of thyroid hormone [121]. It is characterized by morphological [121, 122], biochemical [121] and functional [123] alterations. Asayama and Kato [7] analysed various muscular injury models in which ROS are supposed to play a role. These models provide evidence that mitochondrial function and glutathione-dependent antioxidant system are important for maintenance of the structural and functional integrity of muscular tissues. Because hyperthyroid muscles show similar biochemical changes which are prevented, at least partly, by the suppression of oxidative metabolism and vitamin E administration, it was proposed that ROS contribute to the muscular injury caused by thyroid hormones [7].

However, it is necessary to note that, although lipid peroxidative damage may partly account for the muscle alterations found in hyperthyroidism, there is no direct evidence for a muscular dysfunction mediated by ROS in hyperthyroid animals.

Alterations of heart electrical activity

Although thyroid hormone plays an important role in the regulation of heart mechanical and electrophysiological properties, disorders of the thyroid gland produce alterations in cardiac function. In particular, tachycardia and other arrhythmias, congestive heart failure and heart enlargement can result from an excess of thyroid hormone. About 3 decades ago, intracellular electrode recording from rabbit sinoatrial node cells [124] demonstrated that thyroid hormone can affect heart rate by direct modulating action potential duration (APD) in pacemakers cells. Thyroid hormone-induced increase in heart rate requires APD shortening in atrial and ventricular fibres in order to allow full electrical and mechanical recovery. Records of the action potential from the atrial cells of rabbit [125] and ventricular preparations from other mammalian species [126–129] showed that these results can be obtained by thyroid hormone-induced alterations in membrane currents. Moreover, in the light of the cellular effects of thyroid hormones, it was also suggested the electrophysiological alterations seen in the hyperthyroid state were induced via the synthesis of proteins that are part of ion channels [128]. This idea was supported by the finding that thyroid hormone increases K^+ channel gene expression in rat ventricle [130] and atrium [131] and decreases L-type calcium channel expression in rat atrium [131].

Changes in membrane currents need not result from synthesis of proteins that are part of ionic channels. Indeed, it is been shown that APD shortening in rat ventricular fibres is elicited by procedures that increase free-radical production, such as hydroperoxide administration [132] and physical exercise [133]. ROS involvement in the thyroid hormone-induced modifications in electrical properties of the sarcolemmal membrane, suggested by the similarity between the changes in action potential configuration seen in hyperthyroidism and the aforementioned conditions, was demonstrated by the ability of vitamin E, to attenuate the alterations in heart rate and ventricular APD in hyperthyroid rats [134]. It was also shown that the recovery of APD remained incomplete after higher doses of vitamin E and the antioxidant-sensitive shortening of APD was independent of lipid peroxidation [23]. Thus, it was suggested that the effect of the vitamin on membrane currents was due to protection of sulphydryl-containing ion channel proteins [23].

Liver injury

Thyroid hormones play a major role in hepatic lipid homeostasis. Indeed, they increase the expression of apoliprotein A1 [135], an important component of high-density lipoprotein. They also increase the expression of low-density lipoprotein (LDL) receptors on hepatocytes [135] and the activity of lipid-lowering liver enzymes [136], resulting in a reduction in LDL levels. These effects are beneficial in delaying the onset of atherosclerosis. On the other hand, serious hepatobiliary complications, including centrilobular hepatic necrosis, intrahepatocytic cholestasis and cirrhosis, are associated with untreated hyperthyroidism [137].

It is not clear whether features seen in hyperthyroid liver are secondary to the systemic effects of thyroid hormones or result from direct toxic effects of thyroid hormones on the liver. However, the finding that T_3-induced liver oxidative stress is associated with significant increases in efflux of GSH, lactate dehydrogenase and protein from the liver into the sinusoidal space [109] has suggested that thyroid hormone may destabilise hepatic plasma membranes, possibly via the enhancement in lipid and protein oxidation.

Increase in tissue susceptibility to stressful conditions

Although the pathophysiological consequences of increased oxidative damage in hyperthyroid tissues are not yet fully elucidated, thyroid hormone-induced biochemical changes certainly predispose tissues to free-radical-mediated injury.

Potentation of xenobiotic hepatotoxicity

It is well established that the hepatotoxicity of a variety of substances such as lindane [138], halothane [139], isoflurane and enflurane [140], carbon tetrachloride [141] and chloroform [142], which seems to be linked to the development of an oxidative stress condition, is enhanced by hyperthyroid state.

The mechanism for liver toxicity of anesthetic agents such as isoflurane, enflurane and halothane in hyperthyroid state has been scarcely investigated. Conversely, more information is available on possible mechanisms of thyroid hormone potentiation of the hepatotoxicity of lindane, a substance used as an insecticide and disinfectant in agriculture, which also is a component of some lotions, creams and shampoos against parasites. Lindane was found to induce the liver microsomal cytochrome P450 system and enhance microsomal superoxide [143] and cytosolic $NO^{\bullet}$ [144] generation. Lindane also increased lipid peroxidation and altered some antioxidant mechanisms of hepatocytes, including decreased SOD and CAT activities [143]. These changes related to oxidative stress were dose [145] and time [143] dependent, and coincided with the onset and progression of morphological lesions [143]. Studies concerning the effects of thyroid hormone on lindane hepatotoxicity showed that combined lindane-T_3 administration increased ROS production [145, 146], and GSH [146] and α-tocopherol [147] depletion to an extent that exceeded the sum of the effects elicited by the separate treatments, concomitantly with extensive liver necrosis. In the whole, these data support the view that the increased liver susceptibility to lindane intoxication in hyperthyroidism depends on potentiation of tissue oxidative stress. This effect may be conditioned by an enhanced respiratory burst activity due to Kupffer cell hyperplasia and polymorphonuclear leukocyte infiltration [146], in addition to the increased ROS production in parenchymal cells.

Potentiation of ischemia-reperfusion injury

Although restoration of blood flow is necessary to salvage ischemic tissues, additional insult may occur during reoxygenation and contribute to ischemia-reperfusion injury [148]. Studies of both free-radical production and protective effect of various antioxidants in ischemia-reperfusion systems have suggested that tissue dysfunction during reperfusion results from free-radical generation and oxidative damage [1]. Therefore, ischemia-reperfusion constitutes a model of oxidative injury adequate to test the view of a reduced capability of hyperthyroid tissues to face an oxidative challenge.

Studies of rat liver have shown that hyperthyroid tissues subjected to ischemia reperfusion exhibited elevation in the TBARS/GSH ratio and sinusoidal lactate dehydrogenase efflux largely exceeding the sum of effects elicited by hyperthyroidism or ischemia-reflow alone [149]. These results indicate that the concurrence of the hepatic mechanisms underlying thyroid hormone-induced oxidative stress and ischemia-reperfusion exacerbates liver injury, which appears to be mediated by potentiation of the prooxidant state of the organ.

Investigation has also been performed on functional recovery from *in vitro* ischemia-reperfusion of rat hyperthyroid hearts [150]. Thus, it was shown that, during reperfusion following 20-min ischemia, hyperthyroid hearts displayed significant tachycardia. Furthermore, they exhibited lower recovery of left ventricular developed pressure (LVDP) and maximal rate of developing left ventricular pressure (dP/dtmax), associated with more extensive peroxidative processes compared with euthyroid hearts. Tachycardic response and increase in lipid peroxidation were prevented by treatment of hyperthyroid rats with vitamin E. This suggested that the tachycardic response to reperfusion following chronic T_3 treatment was associated with a reduced capability of the heart to face oxidative stress. Subsequently, evidence was found for $NO^{\bullet}$ involvement in reperfusion-induced dysfunction of hyperthyroid hearts. Indeed, perfusion with the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) prevented tachicardic response to ischemia-reperfusion of hyperthyroid hearts, thus increasing their inotropic recovery up to the euthyroid level [61].

Effects of prolonged exercise on function of hyperthyroid tissues

Studies conducted during the past 3 decades have shown that acute long-term exercise results in an oxidatively stressful environment in the body. Early studies on rats

conducted by Dillard et al. [151] displayed increased breath production of pentane, a by-product of lipid peroxidation, following exercise. Subsequent studies showed elevated levels of lipid peroxidation markers in various tissues, including skeletal [152–154] and cardiac [154, 155] muscles, and liver [152–154]. Direct detection of free radicals by electron spin resonance was also reported in such tissues following exhaustive exercise [152, 155]. Thus, acute exercise can be used to test the idea that thyroid hormone decreases the effectiveness of various target tissues to oppose oxidative challenge.

The effects of prolonged swimming and severe hyperthyroidism on indices of oxidative damage and respiratory capacity of rat tissue homogenates were recently investigated [156]. It was found that both treatments increase hydroperoxides and protein-bound carbonyl levels. Moreover, the highest increases, which were associated to greater reduction in exercise endurance capacity, were found when hyperthyroid animals were subjected to exercise. Aerobic capacity, evaluated by cytochrome oxidase activity, was not modified by exercise, which, conversely, affected the rates of oxygen consumption of hyperthyroid preparations. These results seem to confirm the higher susceptibility of hyperthyroid tissues to oxidative challenge because the opposite changes in respiratory rates during state 4 and state 3 likely involve oxidative modifications of components of mitochondrial respiratory chain, different from cytochrome aa_3.

Role of mitochondria in tissue dysfunctions

Mitochondria are essential for respiration and oxidative energy production in aerobic cells, where they are also required for multiple biosynthetic reaction pathways. Therefore, a disturbance of mitochondrial function due to damage of various mitochondrial components can lead to impaired cell function and even cell death.

Mitochondrial oxidative damage and the antioxidant defence system

Mitochondria are, at the same time, the main site of production and the main target of ROS, so that it is to be expected that increases in ROS production result in enhanced mitochondrial component damage. There are few studies dealing with the effects of altered thyroid states on mitochondrial oxidative damage. However, such studies have shown a general increase in oxidatively damaged lipids [12, 13, 69, 96] and proteins [13, 96] in mitochondria from hyperthyroid tissues.

A larger number of studies is available on the effects of altered thyroid states on the antioxidant status of mitochondria. However, most of these studies concern the changes produced by thyroid hormone in the mitochondrial levels of individual antioxidants.

Determinations of mitochondrial GSH levels showed that such levels decrease in rat liver after 10 days of T_3 treatment [12] and in liver, but not in brain [157] after 6 days of T_4 treatment. On the other hand, it was also shown that 3-day T_3 administration to PTU-treated rats restores normal levels of GSH in liver mitochondria [13]. This result, obtained following short-term hormonal treatment, agrees with previous studies showing that mitochondrial GSH is depleted more slowly than cytosolic GSH [158–160]. The ability of mitochondria to conserve GSH during tissue depletion is due to a transport system for GSH that is located on the inner mitochondrial membrane and is stimulated by ATP [161].

Some information is available regarding the effects of thyroid hormone on vitamin E and Coenzyme Q, present at relatively high concentrations in the inner mitochondrial membrane, where they reduce free radical-initiated lipid peroxidation [162, 163]. Early studies showed that hyperthyroidism increases the content of ubiquinone [97, 164], but does not modify vitamin E content in liver mitochondria [12]. More recently, the increase in ubiquinone content and lack of changes in vitamin E content, besides being confirmed in the liver, has also been shown in cardiac and skeletal muscle from hyperthyroid rats [96]. As regards antioxidant enzymes, it was reported that hyperthyroidism increases the mitochondrial activities of Mn-SOD, CAT, GPX, GR and GST, while it did not modify that of Cu, Zn-SOD in rat liver [13]. Conversely, CAT activity remained unchanged, while SOD activity decreased in heart mitochondria from hyperthyroid rats [165].

The aforementioned results indicate that thyroid hormone differently affects both levels of scavengers and activities of antioxidant enzymes. Thus, for more conveniently evaluating hyperthyroidism effects on the antioxidant status of mitochondria, preparations of these organelles were challenged *in vitro* with specific free radicals [96]. Using such an approach, it was shown that the overall antioxidant capacity of mitochondria decreases in hyperthyroid tissues such as liver, heart and muscle [96]. This indicates that, despite the unbalanced and sometimes opposite changes in the individual components of the mitochondrial antioxidant defence system, the global efficacy of such a system is substantially reduced, thus contributing to increased oxidative damage observed in mitochondria from hyperthyroid tissues.

Mitochondrial susceptibility to oxidative challenge

It is conceivable that thyroid hormone-induced reduction in the global efficacy of the mitochondrial antioxidant defence system can also increase the susceptibility of mitochondria to oxidants. However, other thyroid hormone-induced changes in biochemical characteristics of the mi-

tochondria, such as levels of polyunsaturated fatty acids in mitochondrial membrane phospholipids and content of high and low molecular weight Fe^{2+} complexes, can modify their susceptibility to oxidants and the extent of oxidative damage they suffer when subjected to an oxidative challenge.

Because T_3 administration to hypothyroid rats has been shown to increase total unsaturation of the mitochondrial membrane fatty acids [166], it was expected that lipid peroxidation was elevated in mitochondria of hyperthyroid tissues subjected to oxidative attack. In fact, it was found that the *in vitro* induction of TBARS by oxidants, such as tert-butyl hydroperoxide, in mitochondria from hyperthyroid rats was very high when compared with euthyroid rats [13].

Furthermore, analysis of levels of light emission, resulting from *in vitro* exposure of mitochondrial preparations to H_2O_2, indicated that susceptibility of mitochondria to oxidative challenge is higher in hyperthyroid than in euthyroid tissues, including liver [12, 96], heart and muscle [96]. These results are consistent with the observation that thyroid hormone increases mitochondrial cytochrome content in several tissues, including liver [95, 167] and heart [94].

Mitochondrial damage and tissue dysfunction

To date it is widely accepted that ROS-inflicted damage to mitochondria results in their decreased ability to synthesise ATP, with a consequent lowering in homeostatic ability of tissues to adapt to the destabilising effects of external and internal stresses.

A clear demonstration of this theory has been provided by investigating the role of mitochondria in myocardial ischemia-reperfusion injury. Although ROS may be produced through the operation of xanthine oxidase, which is activated during hypoxia [168, 169], the respiratory chain represents a major source of oxygen radicals during reperfusion of ischemic myocardium [170, 171]. It has also been proposed that ROS generation is induced by interaction with ubisemiquinone, which accumulates in ischemic mitochondria as a result of respiratory chain inhibition [172]. This ROS generation ends rapidly as the autoxidizable electron carriers of the respiratory chain are reoxidized, but it is long-lasting in mitochondria that have accumulated large amounts of reducing equivalents. Thus, the severity of reperfusion-induced oxidative damage and mitochondrial dysfunction might increase with the duration of ischemia. In fact, in early 1975 it was found that impairment of mitochondrial function was enhanced when coronary occlusion periods were increased [173]. Subsequently, it was found that in mitochondria from reperfused hearts, the extent of peroxidative processes increases gradually with the ischemia duration [174], whereas whole antioxidant capacity and resistance to oxidants exhibit significant decreases, likely due to GSH depletion, only after reperfusion following 45 min of ischemia. The above changes were well related to a gradual decline in mitochondrial respiration, which reflected damage to electron transport chain components. Moreover, it was shown that, after reperfusion of ischemic heart, functional recovery of the tissue was inversely correlated to mitochondrial derangement [174], supporting the idea that heart performance is strongly conditioned by mitochondrial functionality. Further support was provided by the observation that protection by antioxidant pretreatment of mitochondrial function is associated with decreased impairment of cardiac function following ischemia-reperfusion [175].

It was expected that the low recovery from ischemia-reperfusion injury of hyperthyroid hearts would depend largely on the reduced ability of mitochondria to regenerate ATP. In fact, it was found that ischemia-reperfusion of hyperthyroid hearts led to both higher impairment of mitochondrial respiration and lower functional recovery compared with euthyroid controls [176]. Moreover, analysis of other reperfusion-linked mitochondrial changes showed that the functional decline of mitochondria during reoxygenation was due to oxidative stress, which was more severe in hyperthyroid hearts [176].

The mechanisms by which ROS mediate the decline in mitochondrial function during reperfusion are not yet entirely understood. However, whatever these mechanisms may be, they must be able to explain why the extent of mitochondrial dysfunction is dependent on the thyroid state. Nitric oxide and its potent oxidative derivative peroxynitrite are putative species responsible for altered mitochondrial function in myocardial ischemia-reperfusion. Inhibition of mitochondrial function by both $NO^{\bullet}$ [177, 178] and peroxynitrite [179] and NOS stimulation upon ischemia-reperfusion [180] have been reported. Furthermore, $NO^{\bullet}$ generated on reperfusion causes mitochondrial dysfunction, damaging complexes I and II of the respiratory chain [181]. These results suggest possible implication of such a substance in altered mitochondrial function in ischemia-reperfusion of hyperthyroid heart.

To throw light on this matter, changes in mitochondrial function following ischemia-reperfusion of hearts from euthyroid and hyperthyroid rats in the presence and absence of L-NNA were compared [62]. The results of this study support the idea that L-NNA improves functional recovery of hyperthyroid hearts by protecting their mitochondria from oxidative damage linked to reperfusion-induced $NO^{\bullet}$ overproduction. Indeed, mitochondrial nitrotyrosine levels were remarkably higher in hyperthyroid than in euthyroid hearts. The differences were reduced when heart perfusion was performed in the presence of L-NNA. Furthermore, the changes in nitrotyrosine levels were associated with opposite changes in mitochondrial function.

Role of mitocondria in tissue rescue from excessive dysfunction

The mechanism which, during reperfusion, causes tissue oxidative damage and dysfunction appears to be a positive feedback loop. Indeed, the concomitance of reflow-mediated perturbations, such as NOS activation and increased ROS production, which have synergistic effects, should enhance oxidative stress and mitochondrial dysfunction. The concurrence of higher ROS generation and NOS activity, together with the reduced efficacy of the mitochondrial antioxidant defence system associated with hyperthyroidism, should increase the extent of mitochondrial dysfunction and tissue derangement. It is conceivable that similar mechanisms underlie the high susceptibility of hyperthyroid tissues to stressful conditions. On the other hand, recent results suggest that, in hyperthyroid tissues, mitochondrial systems able to provide protection against excessive tissue dysfunction are operative.

Mild uncoupling

As it is well known, according chemiosmotic theory, the fall in the potential energy of the electrons which flow through the components of the mitochondrial respiratory chain is used to pump protons from the mitochondrial matrix to the intermembrane space, against their electrochemical gradient. Then, the protonmotive force, produced by H^+ pumping, drives protons back into the matrix through mitochondrial ATP synthase, leading to ATP synthesis. It is also known that lipid-soluble weak acids, which can cross the mitochondrial membrane in a protonated or unprotonated state, are able to dissipate the protonmotive force, thus allowing electron transport to proceed without coupled ATP synthesis. These substances are defined as uncoupling agents. On the other hand, even in the absence of such agents, the inner membrane of isolated mitochondria exhibits a finite proton conductance, which allows a leak of protons back into the matrix, thus reducing the link between oxidation and phosphorylation. Skulachev [182] proposed that the prevention of large increases in the proton electrochemical gradient when ADP is not available represents a mechanism (mild uncoupling) which reduces ROS production by the respiratory chain and consequent oxidative damage. Free fatty acids are good candidates for the role of mild uncoupling, but other components of the mitochondrial membrane, such as members of the mitochondrial transporter superfamily, the uncoupling proteins (UCPs), or ATP/ADP and aspartate/glutamate antiporters, could be involved [183].

Recently, it was found that in skeletal [68] and cardiac [69] muscles state 4 respiration significantly increases without any respiratory control ratio (RCR) change in the transition from hypothyroid to hyperthyroid state. These results are consistent with a previous finding that thyroid hormone-induced increase in respiration is caused by alterations in both the proton leak and phosphorylating systems without significant alteration in RCR [184, 185]. It is possible that this mechanism is mediated by the uncoupling protein-2 (UCP2) and the uncoupling protein-3 (UCP3), whose expression in skeletal [186, 187] and cardiac muscles [188, 189] seems to be regulated by thyroid hormone. Some reports suggest that UCP2 [92] and UCP3 [190] prevent ROS formation by modifying the coupling of mitochondrial respiration to ADP phosphorylation. Such a role seems to require coenzyme Q as an obligatory cofactor [191]. If so, the upregulation of UCPs by thyroid hormone, in addition to the increase in the coenzyme Q content, could provide hyperthyroid muscles with a protection against excessive oxidative damage.

Turnover of mitochondrial proteins

The triiodothyronine-induced decrease in the capacity of mitochondria to withstand oxidative challenge, leading to increased free-radical attack to their components, may have profound implications for the protein turnover. Several observations confirm the proposition that the oxygen radical-mediated oxidation of proteins is a marking step in their turnover. Iron release from hemoglobin and myoglobin induced by excess hydrogen peroxide in model systems [192] has shown that oxidation of hemoproteins can lead to their degradation through a mechanism involving distruption of the heme ring. However, at high concentrations, ROS may play a regulatory role in turnover of macromulecules by causing direct fragmentation of proteins [193]. More frequently, reactions of free radicals with proteins result in the oxidation of various amino acid side chains [194]. A possible consequence of this process is the formation of large protein aggregates, which are toxic to cells if allowed to accumulate. In cells under oxidative stress the formation of large aggregates of oxidatively damaged proteins can be prevented by their rapid elimination. It is well known that the oxidative modification of proteins makes them more susceptible to proteolysis [195, 196]. Thus, they are recognised and completely degraded by proteases and then replaced by protein molecules synthesised de novo [197].

Mammalian cells possess three major proteolytic systems, lysosomal cathepsins [198], calcium-activated calpains [199] and proteasome [200]. It is well established that, after oxidant exposure, proteasome is largely responsible for increased protein degradation in the cytoplasm, nucleus and endoplasmic reticulum of eukaryotic cells [201].

Evidence accumulating over the last 2 decades has demonstrated the existence in mammalian mitochondria of a specific system of proteolytic enzymes. Once, mitochondrial proteins were believed to be removed by lyso-

somes through autophagy of the whole organelle. However, the finding that different mitochondrial proteins turned over at different rates [202] suggested that mitochondria possess a proteolytic pathway which mediates the selective degradation of mitochondrial proteins. They also contain a proteolytic system able to recognise and selectively degrade oxidatively modified proteins [203, 204], thus defending against the accumulation of oxidized proteins. Evidence shows that the expression of a mitochondrial matrix protease, the Lon protease, which may play a key role in this important process [205], is enhanced by thyroid hormone [206]. These results suggest that thyroid hormone is able to carry out a double action on mitochondrial proteins. On one hand, increasing free-radical production by mitochondria and decreasing the ability of such organelles to withstand oxidative challenge should lead to increased damage of mitochondrial proteins. On the other hand, enhancing the level of Lon expression and protease activity should accelerate the removal of oxidatively damaged proteins and should limit mitochondrial dysfunction.

This idea is consistent with early observations that mitochondrial protein turnover is modulated by thyroid hormone [207–209].

Mitoptosisis

During experimental hyperthyroidism there is an increase in total tissue Ca^{2+} concentration, [210–212], together with a greater Ca^{2+} storage capacity in the cell [212]. In presence of Ca^{2+}, oxidative alterations of protein thiols of mitochondrial inner membrane promote the formation of non-specific mitochondrial channels, called the permeability transition pores (PTPs) [213], that lead to mitochondrial swelling. The dependence of Ca^{2+}-induced PTP opening on oxidative alteration of the inner membrane represents a link between oxidative challenge of mitochondria and their susceptibility to swelling. This is supported by finding that liver mitochondria from hyperthyroid rats appear swollen in situ [214], and, when isolated, they exhibit high susceptibility to Ca^{2+}-induced permeabilization of the inner membrane [96, 215], a characteristics which is shared with mitochondria of other hyperthyroid tissues [96].

The susceptibility to permeabilization seems to have important implications for the dynamics of the mitochondrial population and the regulation of cellular ROS production. It is long known that the mitochondrial population is heterogeneous with regard to its sedimentation characteristics, chemical makeup and enzymatic activities [216, 217]. Fatterpaker et al. [207] reported that heavy, light and fluffy mitochondrial fractions, obtained by differential centrifugation, exhibited differences in their stability and the activities of some membrane-bound enzymes in a stress condition caused by carbon tetrachloride administration. More recently, studies on three liver mitochondrial fractions, resolved by differential centrifugation, suggested that the light fractions, with low respiratory activity, contain transitional forms in the process of development into the heavy mitochondrial structures with high respiratory activity [72, 218]. The heavy fraction also exhibits the lowest antioxidant level [12, 72, 218] and the highest rates of H_2O_2 production and susceptibility to Ca^{2+}-induced swelling [72]. However, biochemical and morphological studies have shown that, together with neo-formed mitochondria, the lightest fraction contains disrupted mitochondria which exhibit scarce respiratory capacity [218, 219]. These mitochondria seem to come from the degradation of heavy mitochondria, because conditions leading to increased ROS production and Ca^{2+} overload, such as hyperthyroidism [12], exercise [149] and ischemia-reperfusion [62, 165], decrease the amount of heavy mitochondria and increase that of damaged mitochondria in light fraction from rat tissues. It has been proposed that this phenomenon of mitochondrial destruction mediated by PTP opening, called mitoptosis [183], represents a line of antioxidant defence [220]. It does indeed seem that purifying the mitochondrial population from ROS-overproducing mitochondria protects cells from the harmful side effects of aerobiosis. If true, the mechanism that, in hyperthyroid tissues, enhances the swelling of Ca^{2+}-loaded mitochondria constitutes a negative feedback loop. In fact, the perturbation itself, represented by thyroid hormone-mediated enhancement in ROS generation, should lead to accelerated mitoptosis, thus limiting tissue oxidative damage in hyperthyroid animals.

It is significant that thyroid hormone also increases the rate of mitochondrial biosynthesis [207, 221] and the amount of light mitochondrial proteins [12, 221], thus favouring the substitution of the oldest ROS-overproducing mitochondria, with neoformed mitochondria endowed with a smaller capacity to produce free radicals. Recent research suggests an indirect induction mechanism via the activation of an intermediate factor(s) for the above effect of thyroid hormone. Findings show that expression of the peroxisome proliferator-actived receptor-gamma coactivator-1 (PGC-1), which plays a pivotal role in mitochondrial biogenesis [222], increases not only in response to external stimuli, such as cold temperature or high-calorie diet, but is upregulated by T_3 administration [223, 224]. Moreover, available data suggest that PGC-1 functions as a coactivator for thyroid hormone [225].

Interestingly, the role of mitochondria in tissue rescue from excessive dysfunction is not restricted to the aforementioned processes. Indeed, PTP opening leads to cytochrome c release to cytoplasm, which appears to be an early event in the apoptotic pathway of cell death [226]. This process, which occurs when permeabilization affects a large group of mitochondria, can serve to purify a tissue from cells that produce large amounts of ROS [182].

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