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CHEMILUMINESCENT AND RESPIRATORY RESPONSES RELATED TO THYROID HORMONE-INDUCED LIVER OXIDATIVE STRESS

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Chemiluminescent and respiratory responses were studied in the liver of rats treated with 0.1 mg of triiodothyronine (T_3)/kg for 1 to 7 days. Hyperthyroidism resulted in significant increments in the spontaneous chemiluminescence of the *in situ* liver in animals exhibiting a calorigenic response. Microsomal NADPH-dependent oxygen uptake was enhanced by T_3 treatment for 2 days, an effect that was completely abolished by the antioxidant cyanidanol. A similar microsomal antioxidant-sensitive respiratory component was observed in this situation after the addition of t-butyl hydroperoxide (t-BHP). However, basal rates of microsomal oxygen uptake and light emission in liver homogenates and microsomes were decreased by t-BHP, probably related to thyroid hormone-induced diminution in the content of cytochrome P-450 (Fernández *et al.*)[1] In addition, liver superoxide dismutase and catalase activities as well as the total content of glutathione were depressed by T_3. These results indicate that the calorigenic response in the hyperthyroid state is accompanied by the development of an hepatic oxidative stress characterized by enhanced spontaneous chemiluminescence, enhanced NADPH-dependent microsomal respiration and a decreased antioxidant cellular activity.

KEY WORDS: Liver, oxidative stress, thyroid hormone, chemiluminescence, oxygen uptake.

INTRODUCTION

Studies on the effect of thyroid administration (triiodothyronine, T_3) on rat liver microsomal functions, have indicated that the calorigenic effect of T_3 is accompanied by the stimulation of cellular reactions involving the univalent reduction of oxygen, as evidenced by increments in the rate of superoxide radical ($O^{\cdot}_2$) generation and NADPH oxidase activity.[1] Concomitantly, lipid peroxidative indexes such as liver malondialdehyde accumulation and spontaneous chemiluminescence *in vitro* were significantly enhanced by T_3 treatment, suggesting the development of an oxidative stress condition in the tissue.[1] In line with these findings, Fe^{2+}-induced light emission by rabbit liver mitochondria was found to be increased in hyperthyroid animals compared to controls,[2] while that of hypothyroid rabbits was depressed.[3]

Since the attainment of an experimental hyperthyroid state is characterized by an enhancement of the hepatic respiration[1,4,5] with a lipid peroxidative component,[1] this

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work evaluates the possible relation between T_3-induced calorigenesis and the generation of excited species in the liver. For this purpose, chemiluminescence of the *in situ* rat liver[6] was determined in rats treated with T_3 and was correlated with light emission and oxygen uptake measurements in microsomal fractions. Cellular antioxidant defense mechanisms were also explored by assessing the activity of superoxide dismutase (SOD),glutathione peroxidase (GSH-Px) and catalase (CAT), together with the content of hepatic glutathione.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 240–340 g and fed *ad libitum*, received daily i.p. injections of Na–L–T_3 at a dosage of 0.1 mg of T_3/kg body weight for 1 to 7 consecutive days. Control rats were injected with an equivalent volume of the T_3 diluent (0.1 N NaOH). The hyperthyroid conditions was assessed by measuring the rectal temperature [control rats, 38.3 $\pm$ 0.2°C (n = 11); T_3-treated rats, 38.9 $\pm$ 0.06 (n = 11); $P < 10^{-5}$]. All determinations were carried out 24 hr after the last injection.

In vivo experiments

Chemiluminescence of the *in situ* rat liver was studied after 1,3,5 and 7 days of T_3 treatment. The animals were anesthetized with Nembutal (75 mg/kg i.p.) and abdominal cavity was opened and covered with aluminium foil, allowing exposition of only the liver.[7] Determinations were done with a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, P.A., U.S.A.) according to Videla *et al.*,[7] and the results were expressed as cps/cm^2 of surface area.

In vitro experiments

Determinations of hepatic *in vitro* chemiluminescence were performed in liver homogenates and mitochondrial and microsomal fractions. For this purpose, the livers of Nembutal anesthesized animals were perfused *in situ* with a solution containing 150 mM KCl and 5 mM Tris pH 7.4 and chopped and homogenized (10% w/v) in the same buffer. Mitochondrial and microsomal fractions were prepared according to conventional procedures[8] by centrifuging the post-nuclei (27000 g $\times$ 15 min at 4°C) and the post-mitochondrial supernatants (105000 g $\times$ 60 min at 4°C), respectively. Both pellets were resuspended in the Tris/KCl buffer described above. Chemiluminescence was determined in the presence of 3 mM tert-butyl hydroperoxide (t-BHP) with the fractions diluted to 1 mg of protein/ml (homogenates and mitochondria) or 0.25 mg/ml (microsomes) with a medium containing 150 mM KCl, 5 mM Tris and 1 mM EDTA, pH 7.4. Microsomal oxygen uptake in the absence or presence of 2 mM cyanidanol (CN) was evaluated according to the polarographic method described by Foster,[9] in a medium containing 120 mM sodium phosphate buffer pH 7.4, 0.6 mg of microsomal protein/ml and supplemented either with 0.13 mM NADPH or 0.02 mM t-BHP, at 25°C.

Microsomal thiobarbituric acid reactants (TBAR) were determined according to Buege and Aust.[10]

Hepatic antioxidant parameters

Liver GSH-Px, CAT and SOD activities as well as reduced (GSH) and oxidized (GSSG) glutathione levels were assessed in rats treated with T_3 for 3 days. CAT was determined in the post-nuclei supernatant by measuring the decrease in A_{240}, using a reaction medium containing 50 mM phosphate buffer, pH 7.3, 2 mM hydrogen peroxide, 1% Triton X-100 and 0.1 to 0.3 mg of protein/ml. The obtained k value was divided by the second order reaction constant for pure catalase[11] ($k = 4 \times 10^7 M^{-1} s^{-1}$) and catalase content was expressed as μmol/g of tissue. Total GSH-Px and SOD activities were determined in the post-microsomal supernatant. GSH-Px was evaluated after NADPH oxidation at 340 nm[12] in a reaction medium consisting of 150 mM KCl, 5 mM Tris, pH 7.4, 0.17 mM GSH, 0.2 units/ml GSH-reductase, 0.1 mM NADPH and 0.5 mM t-BHP. SOD was determined by measuring the inhibition of the rate of adrenochrome formation in a reaction medium containing 1 mM epinephrine and 50 mM glycine pH 10.2.[13] The determination of GSH and GSSG was performed in supernatants from 2% (w/v) liver homogenates, prepared in 0.5 N $HClO_4$, centrifuged at 2,500 g for 10 min at 4°C and neutralized with 1.75 M K_3PO_4.[14] GSH was determined using methylglyoxal and glyoxalase I at 240 nm and GSSG was determined using NADPH and glutathione reductase at 340 nm.[15] Protein was measured according to Lowry *et al.*[16] All results are expressed as mean ± S.E.M. and the statistical significance of the differences between mean values was assessed by Student's t-test for unpaired results. All reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Determination of the spontaneous chemiluminescence of the *in situ* liver from hyperthyroid rats revealed significant increments over controls, with a maximal effect (155% increase) elicited after 3 days of treatment (Figure 1). This organ-specific and organ-non-invasive assay is related to both oxy- and organic-peroxy radicals involved in lipid peroxidative reactions,[6,7] and establishes the onset of the liver oxidative stress following T_3 treatment (Figure 1). This chemiluminescence profile coincides with those of the enhancement of both oxygen uptake assessed in liver perfusion studies and *in vitro* lipid peroxidative indexes.[1] The establishment of a pro-oxidative condition in the liver was further explored by measuring the hepatic microsomal respiration, after one and two doses of T_3. As can be seen in Figure 2A, NADPH-dependent microsomal oxygen uptake was significantly enhanced following two doses of T_3 compared to control animals. This is in agreement with the drastic increase in the activity of NADPH-cytochrome P-450 reductase observed in hyperthyroidism,[17,18] which might explain the higher rates of microsomal NADPH oxidation and $O^{\cdot}_2$ generation previously reported.[1,19] Furthermore, the addition of the antioxidant flavonoid cyanidanol[20] to microsomes supplemented with NADPH was able to inhibit oxygen consumption by 39% ($P < 5 \times 10^{-3}$) (Figure 2B) with respect to determinations carried out using NADPH alone (Figure 2A). The difference in oxygen uptake obtained in the absence and presence of cyanidanol corresponds to the microsomal antioxidant-sensitive respiration (MASR) supported by NADPH (Figure 2, inset), and points out to the free-radical nature of the increase in microsomal oxidative capacity observed after 2 days of T_3 administration. A similar microsomal respiratory

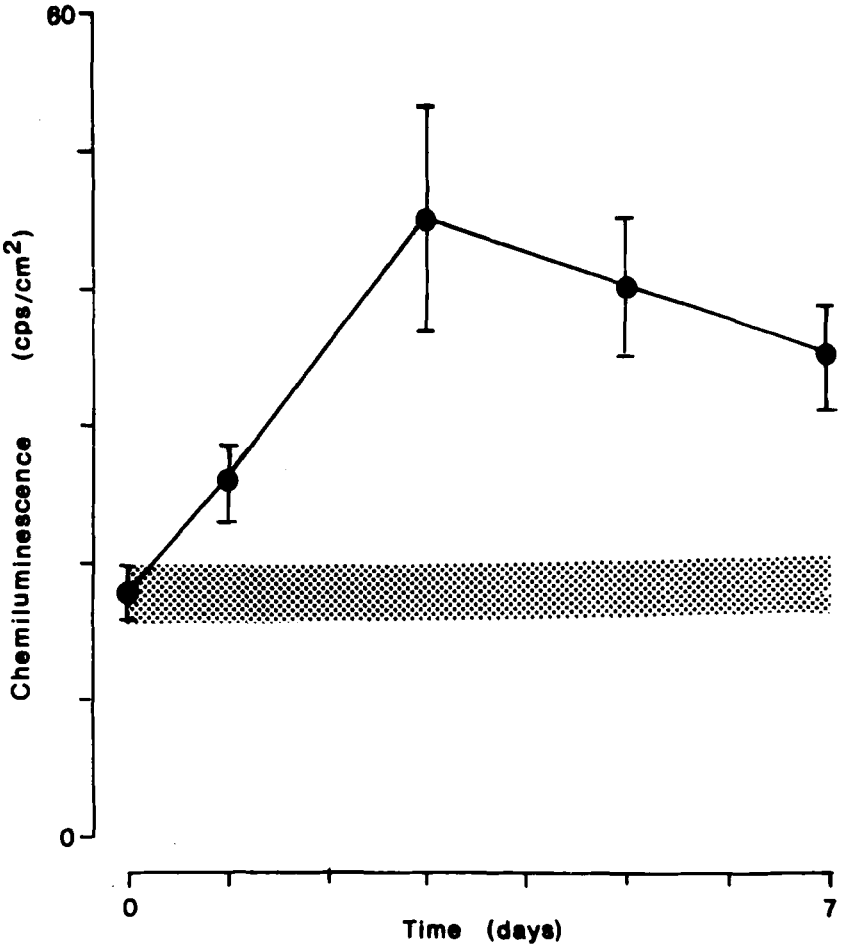

FIGURE 1 Spontaneous chemiluminescence in situ of liver of rats treated with triiodothyronine (T_3) and controls. Rats were given 0.1 mg of T_3/kg i.p. for 1,3,5, and 7 consecutive days and controls received equivalent amounts of T_3 diluent (time zero). Results are the mean ± S.E.M. for 5 animals for experimental time; the shaded area indicates the mean ± 1 S.E.M. for control rats.

component has recently been reported to be induced by lindane treatment[21] and has also been observed in chemically-induced oxidative stress in the perfused rat liver.[22]

Liver microsomes supplemented with t-BHP have been shown to initiate lipid peroxidative processes in the absence of hydrogen donors, depending on the content of cytochrome P-450.[23] Using this assay, microsomal oxygen consumption induced by t-BHP was significantly decreased in rats treated with T_3 for 2 days, compared to controls (Figure 2C). This is in line with the diminution in hepatic content of cytochrome P-450 found in hyperthyroidism[1,17–19] and is reflected in a decreased chemiluminescent response to 3 mM t-BHP observed in liver homogenates [control, 28300 ± 2600 (11) cpm/mg of protein; T_3-treated for 3 days, 20200 ± 1000 (6); $P < 5 \times 10^{-4}$] and microsomes [control, 72300 ± 2600 (9) cpm/mg of protein; T_3-treated for 3 days, 42000 ± 3200 (9); $P < 1 \times 10^{-7}$], without changes in mitoch-

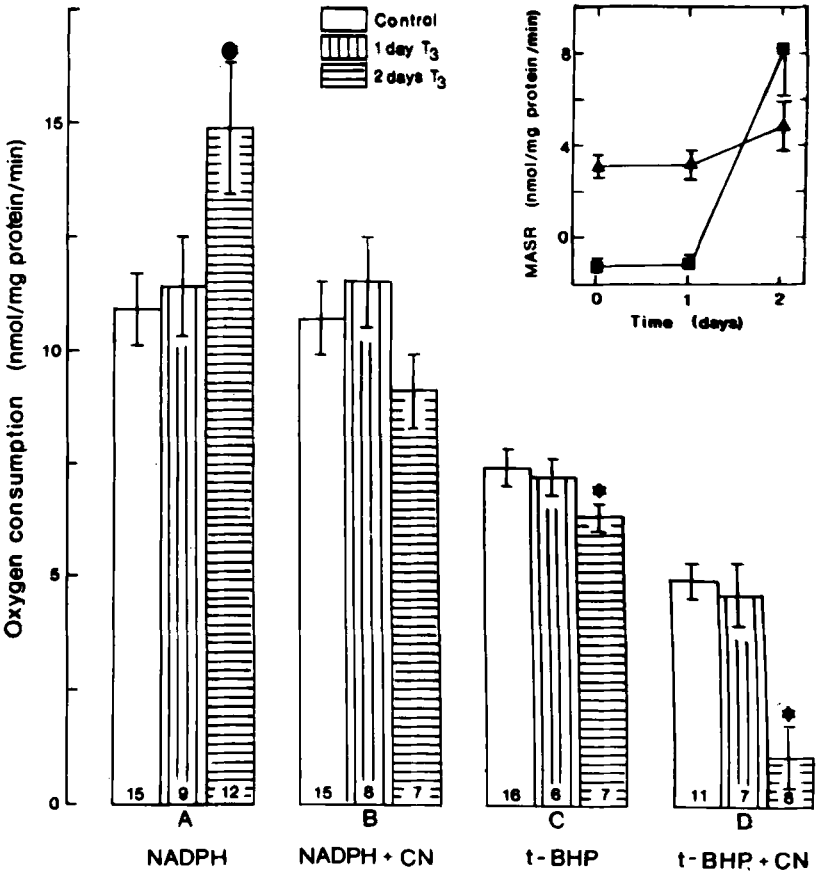

FIGURE 2 Liver microsomal oxygen uptake supported by NADPH or t-butyl hydroperoxide from rats treated with triiodothyronine (T_3) and controls. Rats were given 0.1 mg of T_3/kg i.p. for 1 and 2 days or equivalent amounts of T_3 diluent (controls). Oxygen uptake was initiated by the addition of either 0.13 mM NADPH (A,B) or 2.02 mM t-BHP (C,D), in the absence of presence of 2 mM cyanidanol (CN).

Inset: microsomal antioxidant-sensitive respiration (MASR) dependent on NADPH (■) or t-BHP (▲), calculated by substracting the oxygen uptake found in the presence of CN from that observed in its absence. Results are the mean ± S.E.M. for the number of rats shown at the bottom of the bars. Significance studies were performed by Student's t-test for unpaired data for the effect of T_3-treatment compared to controls: *$P < 5 \times 10^{-3}$.

ondrial fractions [control, 41500 ± 2000 (10) cpm/mg of protein, T_3-treated for 3 days, 38300 ± 2000 (10), N.S.]. In the presence of cyanidanol, t-BHP-induced microsomal oxygen uptake was reduced to a similar extent (Figure 2D) in animals exhibiting comparable levels of cytochrome P-450 (controls and rats treated with T_3 for 1 day),[1] compared to measurements done in the absence of the antioxidant (Figure 2C), thus producing a MASR of about 3.2 nmol/mg of protein/min (Figure 2-inset). Following 2 days of T_3-treatment, however, this respiratory component was enhanced by 54% (Figure 2-inset). Since in this situation the content of cytochrome P-450 is diminished,[1] the increased MASR would rather reflect an endogenous lipid peroxidative component altered by T_3, as microsomal TBAR accumulation is elevated [con-

TABLE I

Effect of administration of triiodothyronine on rat liver antioxidant enzyme activities and glutathione content*

	Parameter	Control	T_3	Effect (%)	P<
A –	Enzyme activity				
	Superoxide dismutase (U/g of liver)	167.0 ± 0.19 (12)	105.2 ± 12.1 (7)	−37	1×10^{-4}
	Catalase (μmol/g of liver)	0.33 ± 0.04 (6)	0.15 ± 0.02 (7)	−55	1×10^{-6}
	Glutathione peroxidase (μmol/g of liver/min)	1.05 ± 0.04 (17)	1.12 ± 0.09 (16)	−7	N.S.
B –	Glutathione				
	GSH (μmol/g of liver)	7.50 ± 0.31 (12)	4.85 ± 0.20 (11)	−35	1×10^{-7}
	GSSG (μmol/g of liver)	0.47 ± 0.06 (12)	0.31 ± 0.03 (11)	−34	3×10^{-2}
	GSH + 2 GSSG (μmol/g of liver)	8.44 ± 0.29 (12)	5.47 ± 0.23 (11)	−35	1×10^{-7}
	GSH/GSSG	16.0 ± 3.3 (12)	15.6 ± 2.6 (11)	−2	N.S.

*Rats were given 0.1 mg of T_3/kg body weight i.p. for 3 days. Values represent the mean ± S.E.M., with the number of animals used in parentheses. Significance studies were carried out with Student's t-test for unpaired data; N.S., not significant.

trol, 1.20 ± 0.19 (12) μmol/mg of protein; T_3-treated for 2 days, 2.59 ± 0.48 (10); $P < 0.005$].

Data presented in Table I show the effect of T_3 treatment for 3 days on the activity of antioxidant enzymes and glutathione content of rat liver. As can be observed, hyperthyroidism was found to significantly reduce the activity of hepatic SOD and CAT, two of the antioxidant defense mechanisms of the cell,[24] without changes of GSH-Px (Table I). In addition, T_3-treatment drastically affected the content of hepatic glutathione, as it significantly diminished GSH and GSSG levels, with a net 35% decrease in total GSH equivalents and no changes in the GSH/GSSG ratio (Table I).

In conclusion, hyperthyroidism in the rat is associated with the development of an oxidative stress condition in the liver tissue. This state is reached by an enhancement in pro-oxidative factors represented by altered chemiluminescent and respiratory responses, together with a decrement in those affording antioxidant protection (decrease in SOD and CAT activities plus glutathione depletion[25]). This phenomenon has recently been proposed as a possible mechanism of injury to heart and skeletal muscle in the hyperthyroid rat[26] and reported to occur in man.[27] In fact, circulating and urinary lipid peroxidative indexes were found to be augmented in hyperthyroid patients compared to euthyroid subjects, being completely reverted to normal after propylthiouracil therapy.[27]

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