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INFLUENCE OF HYPERTHYROIDISM ON SUPEROXIDE RADICAL AND HYDROGEN PEROXIDE PRODUCTION BY RAT LIVER SUBMITOCHONDRIAL PARTICLES

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Administration of daily doses of 0.1 mg of 3,5,3′-triiodothyronine (T_3)/kg body weight for 3 consecutive days to fed rats elicited a calorigenic response in the animals, in concomitance with a 36% increase in the rate of 0_2 consumption by the liver. In these conditions, liver submitochondrial particles (SMP) from T_3-treated rats exhibited marked increases in the rate of superoxide radical generation, both in the presence of NADH (142%) or succinate (152%). Furthermore, liver SMP from hyperthyroid animals released hydrogen peroxide at higher rates than those of euthyroid rats, either under basal conditions or in the succinate-supported process, both in the absence and presence of antimycin-A. It is concluded that the hyperthyroid state in the rat leads to a drastic enhancement in the capacity of liver mitochondria to produce active oxygen species, which correlates with the elevated respiratory rate observed in the intact organ.

KEY WORDS: Hyperthyroidism, superoxide radical, hydrogen peroxide, rat liver submitochondrial particles, thyroid calorigenesis, oxidative stress.

INTRODUCTION

Thyroid calorigenesis in the rat has been shown to produce major molecular changes in the liver, concomitantly with the enhancement in the rate of O_2 consumption, leading to the development of an oxidative stress condition.[1] This condition is related to the increment in the generation of active O_2 species[2] and to decrements in the activity of several antioxidant defense systems of the hepatocyte,[3] with the consequent lipid peroxidative response.[2-5] Stimulatory effects of 3,5,3′-triiodothyronine (T_3) and 3,5,3′,5′-tetraiodothyronine (T_4) on liver microsomal NADPH-dependent pathways have been reported,[1] processes known to involve one-electron transfer reactions.[6] These include drastic increments in the activities of NADPH-cytochrome P-450 reductase[7-9] and NADPH oxidase,[2,7,10] as well as superoxide radical (O_2^-) generation.[2] These observations led to the proposal that O_2 utilization in active O_2 species production and lipid peroxidation[11] might contribute to the enhanced respiratory rate observed in the liver of hyperthyroid rats,[1] as well as in other target tissues for thyroid hormone action,[12] thus determining part of the calorigenic response.[1]

Apart from the microsomal fraction, mitochondria have also been reported to generate active O_2 species, depending on their metabolic state. These include the NADH-supported production of O_2^- by submitochondrial particles (SMP) from bovine heart,[13] as well as hydrogen peroxide (H_2O_2) generation by intact pigeon

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heart and rat liver mitochondria, assayed in state 4.[14] Furthermore, liver mitochondria isolated from rats treated with 2,4-dinitrophenol exhibited a drastic increase in H_2O_2 generation over control values, associated with a reduction in their ADO/O ratios and the induction of a calorigenic state in the animals.[15] These observations, and the development of an antioxidant-sensitive respiratory component by high concentrations of 2,4-dinitrophenol added to perfused rat livers,[16] suggest the involvement of mitochondrial univalent reduction of O_2 in the uncoupled state. Since the oxidative capacity of liver mitochondria is significantly increased by thyroid hormones,[17] in the present work we focused our attention upon the possibility that hyperthyroidism may affect mitochondrial one-electron transfer reactions, by measuring the rates of O_2^- and H_2O_2 generation by rat liver SMP.

MATERIALS AND METHODS

Male Wistar rats weighing 270–300 g and fed ad libitum, received daily ip injections of 0.1 mg of T_3/kg body weight or equivalent volumes of 0.1 N NaOH (T_3 diluent) (controls) for 3 consecutive days. Following 24 h of the last injection, the weight of the animals was comparable in both groups [controls, 303 ± 10 (n = 19) g; T_3-treated rats, 286 ± 12 (n = 17); means ± S.E.]. At this experimental time, serum T_3 levels were measured by the GammaCoat™ [^{125}I]T_3 Radioimmunoassay Kit (Baxter Healthcare Corp., Cambridge, MA), together with the rectal temperature of the animals measured with a thermocouple, as well as the rate of O_2 consumption measured polarographically in perfused livers, as described elsewhere.[2]

The animals were anesthetized with Nembutal (50 mg/kg ip) and the livers were perfused in situ with 200 ml of a cold solution containing 150 mM KCl and 5 mM Tris pH 7.4, prior to the preparation of SMP by ultrasonic oscillation of intact mitochondria, followed by centrifugation at 105,000xg for 60 min at 4°C, as described by Pedersen *et al.*[18] The pellet was resuspended either in MSH buffer (220 mM mannitol, 70 mM sucrose and 0.5 g/l serum bovine albumin, pH 7.3) for O_2^- determinations, or in MST buffer (225 mM mannitol, 75 mM sucrose, 0.2 mM EDTA, 2 mM Hepes and 0.5 g/l serum bovine albumin, pH 7.4) for H_2O_2 measurements. O_2^- production was determined by monitoring the superoxide dismutase-sensitive generation of adrenochrome at 480 nm, using 1 mM epinephrine, 2 μM antimycin-A, 150 U/ml catalase and 0.6 mg/ml of mitochondrial protein, with either 0.1 mM NADH or 5 mM succinate as substrates.[19] H_2O_2 production was measured by monitoring the formation of the compound II between H_2O_2 and added horseradish peroxidase (HRP) (136 U/ml) at 417-402 nm, in an Aminco-Chance dual wavelength spectrophotometer.[20] The reaction was initiated by adding HRP to 0.15 mg/ml of mitochondrial protein in MST buffer (H_2O_2 generation supported by endogenous substrate), with further additions of 4 mM succinate and/or 0.18 μM antimycin-A. In order to evaluate the possible peroxisomal contamination of SMP, H_2O_2 production was measured in the presence of 30 μM urate. All determinations were carried out at 28°C. Proteins were measured according to Lowry *et al.*[21] O_2^- and H_2O_2 generation were expressed as nmol/mg protein/min, and calculations were done using the extinction coefficients of 4×10^2 $M^{-1} cm^{-1}$ and 5×10^4 $M^{-1} cm^{-1}$, respectively. Mitochondrial superoxide dismutase (MnSOD) activity was determined in 0.05 M sodium phosphate buffer pH 7.5 containing 0.1 mM EDTA and 0.15 mM hematoxylin, by measuring the MnSOD-sensitive autoxidation of hematoxylin at 560 nm.[22] The values shown represent the

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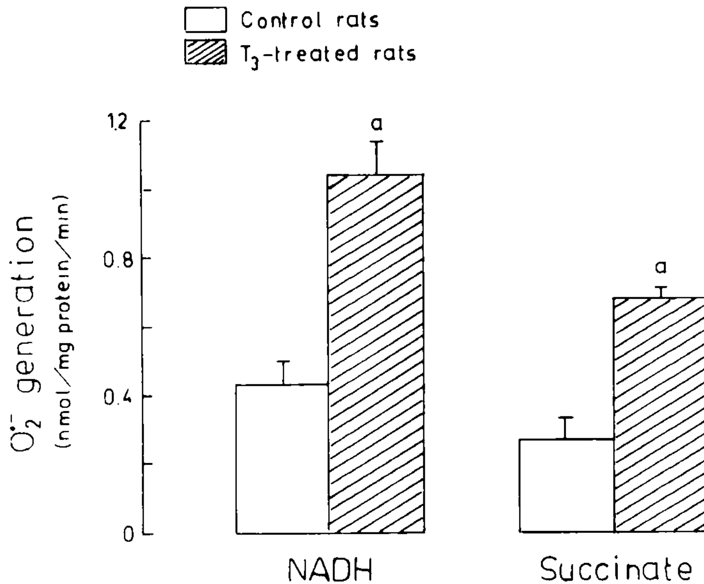

FIGURE 1 Rates of superoxide radical (O_2^-) formation by liver submitochondrial particles (SMP) from control rats and T_3-treated animals. Liver SMP equivalent to 0.6 mg of protein/ml were used. O_2^- generation was determined as the superoxide dismutase-sensitive formation of adrenochrome, in the presence of either 0.1 mM NADH or 5 mM succinate. Values shown represent the means ± S.E. of 4–8 animals per experimental group.[a] $P < 0.05$, compared to controls.

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means ± S.E. for the number of experiments indicated. Significance of differences between mean values was assessed by Student't test for unpaired data. All reagents used were obtained from Sigma Chemical Co. (St Louis, MO).

RESULTS AND DISCUSSION

Administration of daily doses of 0.1 mg of T_3/kg to fed rats for 3 consecutive days resulted in significant increments in the serum levels of the hormone [controls, 48 ± 4 ($n = 5$) ng/dl; T_3-treated rats, 327 ± 42 ($n = 5$); $P < 0.05$], together with an enhanced metabolic rate, as evidenced by the significant increase in the rectal temperature of the animals [controls, 37.5 ± 0.1 ($n = 15$) °C; T_3-treated rats, 38.4 ± 0.1 ($n = 17$); $P < 0.05$]. This T_3-induced thermogenic condition was further reflected in a 36% increase in the rate of O_2 consumption of the liver, assessed in perfusion experiments [controls, 1.95 ± 0.05 ($n = 5$) μmol/g liver/min; T_3-treated rats, 2.65 ± 0.07 ($n = 5$]; $P < 0.05$]. In these conditions, T_3 treatment elicited a significant enhancement in O_2^- generation by liver SMP, using either NADH (142% increase) or succinate (152% increase) as mitochondrial substrates (Figure 1). Since in this situation the activity of MnSOD was not modified [controls, 2.55 ± 0.37 ($n = 6$) U/mg protein; T_3-treated rats, 3.20 ± 0.65 ($n = 8$); not significant], a mitochondrial oxidative stress condition is induced by T_3 treatment. This is evidenced by the significant 49% decrease in the MnSOD activity/NADH-dependent O_2^- generation ratio [controls, 5.9 ± 0.6 ($n = 6$); T_3-treated rats, 3.0 ± 0.5 ($n = 8$);

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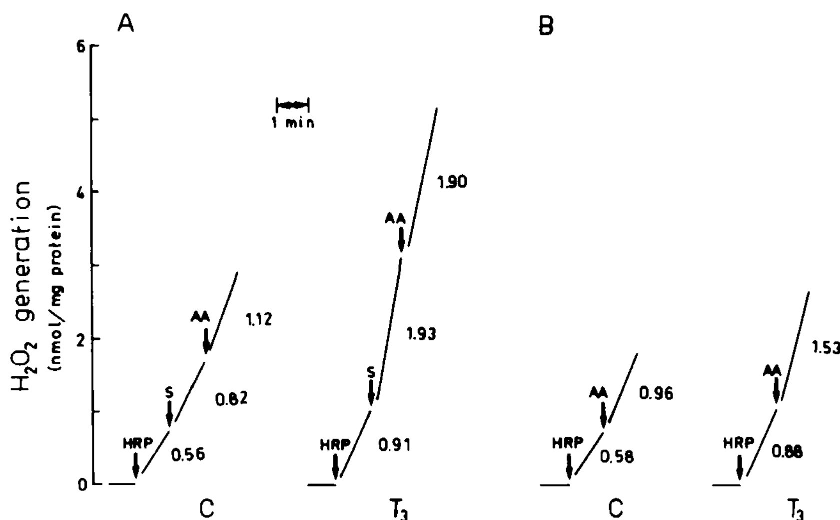

FIGURE 2 Generation of hydrogen peroxide (H_2O_2) by liver submitochondrial particles (SMP) from control rats (C) and T_3-treated animals (T_3). Liver SMP equivalent to 0.15 mg of protein/ml were used. H_2O_2 was determined by the formation of the compound II with added horseradish peroxidase (HRP) (136 U/ml), after [A] succesive additions of 4 mM succinate (S) and 0.18 μM antimycin-A (AA) or [B] in the presence of 0.18 μM AA alone. Values adjacent to the traces correspond to the rates of H_2O_2 formation in nmol/mg protein/min.

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$P < 0.05$], as well as in that of the MnSOD activity/succinate-supported O_2^- production [data not shown].

The rate of O_2^- generation by SMP depended on the substrate added to the preparations, as the ratio of the NADH-dependent production rate to that supported by succinate was 1.60 in control rats and 1.53 in T_3-treated animals (from Figure 1). These results are in agreement with the proposal that the mitochondrial respiratory chain generates O_2^- at two different sites, located near NADH dehydrogenase and in the ubiquinone-cytochrome b area.[13] As O_2^- is considered to represent the stochiometric precursor for mitochondrial H_2O_2 formation,[13] increments in H_2O_2 production would be expected in liver SMP from T_3-treated rats.

Using different experimental conditions to evaluate H_2O_2 generation, liver SMP from hyperthyroid animals were found to produce H_2O_2 at higher rates than those from euthyroid rats (Figure 2). This was evidenced by the 56% increase in the basal H_2O_2 formation observed after HRP addition [controls, 0.58 ± 0.02 (n = 19) nmol/mg protein/min; T_3-treated rats, 0.90 ± 0.08 (n = 17); $P < 0.05$], or in that supported by succinate alone [controls, 0.80 ± 0.05 (n = 8) nmol/mg protein/min; T_3-treated rats, 1.95 ± 0.40 (n = 11); 143% increase; $P < 0.05$] and in the presence of antimycin-A [controls, 1.10 ± 0.14 (n = 6) nmol/mg protein/min; T_3-treated rats, 1.90 ± 0.60 (n = 5); 72% increase; $P < 0.05$] (Figure 2A). Addition of antimycin-A stimulated basal H_2O_2 production by liver SMP in controls rats [from 0.58 ± 0.02 (n = 19) to 0.95 ± 0.09 (n = 5) nmol/mg protein/min ($P < 0.05$)] and in T_3-treated animals [from 0.90 0.08 (n = 17) to 1.52 ± 0.23 (n = 8) nmol/mg protein/min ($P < 0.65$)] (Figure 2B). However, H_2O_2 production dependent on added succinate was significantly enhanced by the addition of antimycin-A in liver SMP from control rats [from 0.80 ± 0.05 (n = 8) to 1.10 ± 0.14 (n = 6) nmol/mg

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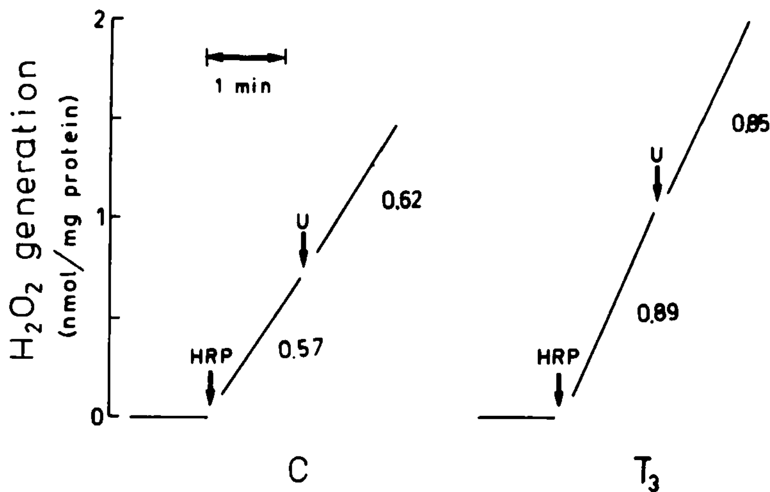

FIGURE 3 Influence of urate on the generation of hydrogen peroxide (H_2O_2) by liver submitochondrial particles (SMP) from control rats (C) and T_3-treated animals (T_3). Liver SMP equivalent to 0.15 mg of protein/ml were used. H_2O_2 was evaluated by the formation of the compound II with added horseradish peroxidase (HRP) (136 U/ml), in the absence and presence of 30 μM urate (U). Values adjacent to the traces correspond to the rates of H_2O_2 formation in nmol/mg protein/min.

protein/min ($P < 0.05$)], but not in those from T_3-treated animals [1.95 ± 0.40 ($n = 11$) versus 1.90 ± 0.60 ($n = 5$) nmol/mg protein/min; not significant] (Figure 2A). This could be explained considering that increments in succinate-dependent H_2O_2 generation in SMP from hyperthyroid rats might be linked to the higher activity of succinate dehydrogenase reported in this condition.[17] In the absence of ADP, addition of succinate would lead to a rapid reduction of the flavoprotein, with a minimal electron flux towards O_2 for H_2O formation and maximal for H_2O_2 production.[23] Thus, since the rate of H_2O_2 generation is likely to be near Vmax values in the hyperthyroid state, the addition of antimycin-A would have no further effect on this parameter. In the experimental design used, H_2O_2 production due to peroxisomal contamination seems unlikely, as no significant effects were seen on the H_2O_2 release by liver SMP from controls [HRP, 0.58 ± 0.02 ($n = 19$) nmol/mg protein/min; urate, 0.64 ± 0.07 ($n = 6$); $P > 0.05$] and T_3-treated rats [HRP, 0.90 ± 0.08 ($n = 17$) nmol/mg protein/min; urate, 0.83 ± 0.11 ($n = 7$); $P > 0.05$], after the addition of urate (Figure 3).

The enhancement in rat liver mitochondrial univalent reduction of O_2 by hyperthyroidism, leading to a greater generation of O_2^- and H_2O_2, could be the result of increments in the content and activity of several respiratory chain components, which determine an increased oxidative capacity in these organelles.[17,24] In addition, the destabilization of one or more components of the mitochondrial respiratory chain could enhance their potential for autoxidation, increasing the production of O_2^- and H_2O_2, as suggested for mitochondria sustaining a reduced respiratory control.[15] The elevated oxidative capacity observed in liver SMP from hyperthyroid rats might determine higher steady-state concentrations of O_2^- and H_2O_2 in the intact organelle, with the subsequent lipid peroxidative response. This view is in agreement with previous data by Marzoev *et al.*,[5] showing an increased iron-mediated chemiluminescent response in isolated liver mitochondria from hyperthyroid

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rabbits, parameter known to be related to free radical-induced lipid peroxidative processes.[25]

In conclusion, hyperthyroidism in the rat leads to a marked enhancement in the mitochondrial generation of active O_2 species, assessed in liver SMP, in addition to the greater O_2 utilization needed to account for the elevated rates of microsomal O_2^- production and cellular lipid peroxidative processes previously reported.[2] In this respect, changes in peroxisomal H_2O_2 generation cannot be discarded, as thyroid hormones are known to induce a distinct peroxisome population in the liver,[26] together with drastic increases in D-amino oxidase activity[26] and in peroxisomal fatty acid β-oxidation,[27] processes related to H_2O_2 formation.[28] However, this aspect has not been evaluated in the hyperthyroid state. The enhancement in O_2 equivalents related to T_3-induced oxidative stress observed in liver subcellular fractions has recently been evaluated in the isolated perfused rat liver, by the addition of either desferrioxamine or allopurinol, which partially inhibited the increase in O_2 uptake induced by T_3.[29] This antioxidant-sensitive respiratory component is enhanced by about 60% by T_3 over control values, and represents either 16–25% of the net increase in the O_2 consumption of the liver elicited by the hormone treatment or 3–5% of the total respiratory rate observed in the liver of T_3-treated animals.[29]

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References

1. V. Fernández and L.A. Videla (1989) Thyroid hormone, active oxygen, and lipid peroxidation. In *Handbook of Free Radicals and Antioxidants in Biomedicine* (J. Miquel, A.T. Quintanilha and H. Weber, eds.), CRC Press Inc., Boca Raton, vol. I, pp. 105–115.
2. V. Fernández, X. Barrientos, K. Kipreos, A. Valenzuela and L.A. Videla (1985) Superoxide radical generation, NADPH oxidase activity, and cytochrome P-450 content of rat liver microsomal fractions in an experimental hyperthyroid state: relation to lipid peroxidation. *Endocrinology*, **117**, 496–501.
3. V. Fernández, S. Llesuy, L. Solari, K. Kipreos, L.A. Videla and A. Boveris (1988) Chemiluminescent and respiratory responses related to thyroid hormone-induced liver oxidative stress. *Free Radical Research Communications*, **5**, 77–84.
4. C. Landriscina, V. Petragallo, P. Morini and G.O. Marcotrigiano (1968) Lipid peroxidation in rat liver microsomes. I. Stimulation of the NADPH-cytochrome P-450 reductase-dependent process in hyperthyroid state. *Biochemistry International*, **17**, 385–393.
5. A.I. Marzoev, A.V. Kozlov, A.P. Andryushchenko and Y.A. Vladimirov (1982) Activation of lipid peroxidation in liver mitochondria of hyperthyroid rabbits. *Bulletin of Experimental Biology and Medicine*, **93**, 269–272.
6. R.W. Estabrook and J. Werringloer (1977) Cytochrome P-450: its role in oxygen activation for drug metabolism. In *Drug Metabolism Concepts*, A.C.S., Symposium Series 44 (D.M. Jerina, ed.), American Chemical Society, Washington, D.C., pp. 1-26.
7. R. Kato and A. Takahashi (1968) Thyroid hormone and activities of drug-metabolizing enzymes and electron transport systems of rat liver microsomes. *Molecular Pharmacology*, **4**, 109–120.
8. P.S. de Araujo, R. de Andrade-Silva and I. Raw (1962) Effect of drugs and hormones on rat liver dimethylaminoazobenzene reductase activity. *Brazilian Journal of Medical and Biological Research*, **15**, 17–28.
9. A.H. Phillips and R.G. Langdon (1956) The influence of thyroxine and other hormones on the hepatic TPNH-cytochrome c reductase activity. *Biochimica et Biophysica Acta*, **19**, 380–382.
10. Y. Israel, L. Videla, A. MacDonald and J. Bernstein (1973) Metabolic alterations produced in the liver

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by chronic ethanol administration. Comparison between the effects produced by ethanol and by thyroid hormones. *Biochemical Journal*, **134**, 523-529.

11. H. Kappus (1985) Lipid peroxidation: mechanism, analysis, enzymology and biological relevance. In *Oxidative Stress* (H. Sies, ed.), Academic Press, London, pp. 272-310.
12. K. Asayama, K. Dobashi, H. Hayashibe, Y. Megata and K. Kato (1987) *Lipid peroxidation and free radical scavengers in thyroid dysfunction in the rat: a possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. Endocrinology*, **121**, 2112-2118.
13. J.F. Turrens and A. Boveris (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochemical Journal*, **191**, 421-427.
14. A. Boveris and B. Chance (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal*, **134**, 707-716.
15. S.E. Dryer, R.L. Dryer and A.P. Autor (1980) Enhancement of mitochondrial cyanide-resistant superoxide dismutase in the livers of rats treated with 2,4-dinitrophenol. *Journal of Biological Chemistry*, **255**, 1054-1057.
16. L.A. Videla (1984) Hepatic antioxidant-sensitive respiration: effect of ethanol, iron and mitochondrial uncoupling. *Biochemical Journal*, **223**, 885-891.
17. K. Sterling (1986) Thyroid hormone action at the cellular level. In *The Thyroid, A Fundamental and Clinical Text* (S.H. Ingbar and L.B. Bravermann, eds.), J.B. Lippincott Co., Philadelphia, pp. 219-233.
18. P.L. Pedersen, J.W. Greenawalt, B. Reynafarje, J. Hullihen, J.L. Decker, J.W. Soper and E. Bustamante (1978) Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. In *Methods in Cell Biology*, Academic Press, New York, vol. 20, pp. 411-481.
19. J.F. Turrens, B.A. Freeman, G.J. Levitt and J.D. Crapo (1982) *The effect of hyperoxia on superoxide production by lung submitochondrial particles. Archives of Biochemistry and Biophysics*, **217**, 401-410.
20. A. Boveris, N. Oshino and B. Chance (1972) The cellular production of hydrogen peroxide. *Biochemical Journal*, **128**, 617-630.
21. O.H. Lowry, N. Rosebrough, A. Farr and R. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
22. J.P. Martin, M. Dailey and E. Sugarman (1987) Negative and positive assays of superoxide dismutase based on hematoxylin autoxidation. *Archives of Biochemistry and Biophysics*, **255**, 329-336.
23. J.F. Turrens, B.A. Freeman and J.D. Crapo (1962) Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. *Archives of Biochemistry and Biophysics*, **217**, 411-421.
24. S.B. Barker (1951) Mechanism of action of the thyroid hormone. *Physiological Reviews*, **31**, 205-243.
25. M.E. Murphy and H. Sies (1990) Visible-range low-level chemiluminescence in biological systems. *Methods in Enzymology*, **186**, 595-610.
26. W.W. Just, F.U. Hartl and H. Schimassek (1982) Rat liver peroxisomes. I. New peroxisome population induced by thyroid hormones in the liver of male rats. *European Journal of Cell Biology*, **26**, 249-254.
27. W.W. Just and F.U. Hartl (1983) Rat liver peroxisomes. II. Stimulation of peroxisomal fatty-acid beta-oxidation by thyroid hormones. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie*, **364**, 1541-1547.
28. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, **59**, 527-605.
29. V. Fernández and L.A. Videla (1993) 3,3',5-Triiodothyronine-induced hepatic respiration: effects of desferrioxamine and allopurinol in the isolated perfused rat liver. *Toxicology Letters*, in press.

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