Brain chemiluminescence and oxidative stress in hyperthyroid rats

Ana M. ADAMO,* Susana F. LLESUY, Juana M. PASQUINI and Alberto BOVERIS

Departamento de Química Biológica-IQUIFIB, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires-CONICET, Junin 956, 1113 Buenos Aires, Argentina

Newborn Wistar rats were made hyperthyroid by injection of tri-iodothyronine and assayed for survival, brain oxygen uptake, brain chemiluminescence and activity of antioxidant enzymes. Brain chemiluminescence was measured (1) by removing the parietal bones or (2) through the translucent parietal bones. Control animals showed a brain chemiluminescence of 130 ± 12 c.p.s./cm² and 99 ± 10 c.p.s./cm² for procedures (1) and (2) respectively. Hyperthyroid rats showed increases in the spontaneous brain photoemission of 46 and 70 % compared with controls, measured by procedures 1 and 2 respectively. The hyperthyroid state did not modify the oxygen-dependent chemiluminescence of brain homogenates. The hyperthyroid animals showed a 30 % increase in the oxygen uptake of brain slices and a dramatic shortening of life-span to about 16 weeks. Superoxide dismutase (the Cu–Zn enzyme), catalase and Se-dependent glutathione peroxidase activities of brain homogenates were increased by 18, 36 and 30 % respectively in the hyperthyroid animals. Isolated brain mitochondria produced 0.18–0.20 nmol of H₂O₂/min per mg of protein in state 4 in the presence of succinate as substrate. No difference was observed between control and hyperthyroid animals. It is concluded that hyperthyroidism leads to hypermetabolism and oxidative stress in the brain. The increased levels of oxygen and peroxy radicals may contribute to premature ageing in these animals.

INTRODUCTION

Neonatal euthyroidism is necessary for the normal development, growth and maturation of the central nervous system (Pasquini *et al.* 1967). The presence of normal levels of thyroid hormones is critical during the period of myelination (Bhat *et al.*, 1979, 1981).

A number of tissues, such as brain, liver, heart and muscle, when isolated from hyperthyroid animals, show increased oxygen consumption (Oppenheimer *et al.* 1979), which could lead, through an increased steady-state level of oxygen intermediates, to the establishment of oxidative stress in the tissue. Fernández *et al.* (1988) have recently reported that lipid peroxidation indexes such as spontaneous chemiluminescence and malondialdehyde level were increased in the liver by tri-iodothyronine treatment, suggesting the development of a condition of oxidative stress. Increased levels of the intermediates of oxygen reduction were singled out by Gerschman *et al.* (1954) as a cellular condition that may lead to premature ageing. The modern view is that O₂^{•-}, H₂O₂ and OH are derived from side reactions of normal oxidative metabolism (Chance *et al.*, 1979) and that increased levels of these reactive oxygen species define a condition of oxidative stress (Sies, 1985) with increased rates of formation of oxygen- and carbon-centred radicals.

Thyroid function seems to have a regulatory role concerning life-span. Rats hypothyroid from birth live longer than their controls (Ooka *et al.*, 1983) and thyroxine administration significantly shortens life-span (Timiras, 1986).

The present work studies some cellular events which occur in the brain during myelination that may contribute to a condition of hypermetabolism and premature ageing. We aim to establish a relationship between tri-

iodothyronine-induced hypermetabolism and the occurrence of a state of oxidative stress.

MATERIALS AND METHODS

Animals

Wistar rats (18 days old), both hyperthyroid and control (22.4 ± 0.5 and 27.8 ± 0.4 g body wt. respectively) of both sexes were used. Newborn rats were made hyperthyroid using the method of Cocks *et al.* (1970), by injecting them subcutaneously with tri-iodothyronine (25 µg at birth; 3 µg on the second day of life and 0.5 and 1.5 µg on alternate days thereafter until the end of the experiment). Control animals received similar volumes (50 µl) of saline solution.

Oxygen uptake by brain slices

Rats were killed by decapitation, brains were dissected out and 0.5 mm tissue slices were cut using a tissue slicer (Harvard Apparatus, Boston, MA, U.S.A.). Slices were kept in Krebs–Ringer bicarbonate buffer, pH 7.3, containing 10 mM-glucose, at 0–2 °C. Oxygen consumption was measured with the brain slices suspended in the same buffer and placed in an adequate cuvette regulated at 30 °C and equipped with a Clark oxygen electrode (Puntarulo *et al.*, 1988).

Determination of chemiluminescence

Brain chemiluminescence *in vivo*. The animals were anaesthetized with urethane 12 % (w/v) (0.1 mg/kg body wt., intraperitoneal) and brain emission was measured in one of two ways: (1) through the translucent parietal bones after exposing the skull, or (2) from the exposed brain, after removing the meninges and the parietal

* To whom correspondence and reprint requests should be addressed.

Fig. 1. Scheme showing positioning and surgical procedures to measure brain chemiluminescence

(a) Measurement through the parietal bones: 1, light guide of the photon counter; 2, aluminium foil. (b) Measurement of the chemoluminescence of the exposed brain after removing the parietal bones. The circle indicates the exposed area.

bones by cutting them out with curved scissors (Fig. 1). In both cases the whole animal was covered with aluminium foil with a cut window, through which chemiluminescence was measured. Determinations were made with a Johnson Foundation photon counter specially adapted for organ chemiluminescence (Boveris *et al.* 1980). Emissions are expressed as counts per second (c.p.s.)/cm² of exposed area.

Homogenate chemiluminescence *in vitro*. Rats were anaesthetized with diethyl ether. A small cut was made in the suprahepatic veins to bleed the animal, while perfusion with an ice-cold 0.9% (w/v) NaCl solution was simultaneously carried out through a needle placed in the left ventricle of the heart. After 15 min of perfusion, animals were decapitated and the essentially blood-free brains were excised and placed on an ice-cold glass plate. The tissues were homogenized in a Teflon-glass homogenizer at 10% (w/v) in ice-cold 0.12 M-KCl/0.5 mM-EDTA/30 mM-phosphate buffer, pH 7.2, and centrifuged at 750 *g* for 10 min at 0–2 °C. The supernatant was used as 'homogenate' to measure spontaneous oxygen-dependent chemiluminescence (Cadenas *et al.*, 1981) in a Packard Tri-Carb model 3320 scintillation counter in the out-of-coincidence mode at room temperature. The homogenate, at a protein concentration of 0.5 mg of protein/ml, was placed in glass vials (diam. 10 mm, height 25 mm) which were then placed in low-potassium glass scintillation vials (diam. 25 mm, height 45 mm) (Boveris *et al.*, 1983). The emission was expressed as c.p.s./mg of protein. Protein concentration was assayed according to the method of Lowry *et al.* (1951).

Assay of the activity of antioxidant enzymes in brain homogenates

Glutathione peroxidase activity was determined by following spectrophotometrically the rate of NADPH oxidation at 340 nm (ϵ 6.22 mM⁻¹·cm⁻¹) in a reaction medium consisting of 0.3 mM-NADPH, 0.17 mM-GSH, yeast glutathione reductase (0.2 units/ml), 50 mM-phosphate buffer, pH 7.3, 0.3–0.5 mg of homogenate protein/ml and either 0.5 mM-t-butyl hydroperoxide or 0.5 mM-H₂O₂ as oxidant. When H₂O₂ was used as oxidant, 20 μ M-sodium azide was added to inhibit catalase. The activity with H₂O₂ was referred to as Se-dependent glutathione peroxidase, and that with t-butyl hydroperoxide was the sum of the activity of the Se-dependent and Se-independent enzymes. The activity was expressed

in nmol of NADPH oxidized/min per g of tissue (Flohé & Gunzler, 1984).

Catalase activity was determined by measuring the absorbance decrease at 240 nm in a reaction medium containing 2 mM-H₂O₂, 1% Triton X-100, 50 mM-phosphate buffer, pH 7.3, and 0.1–0.3 mg of protein/ml. The activity was expressed as equivalent to nmol of catalase/g of tissue by dividing the obtained k' , the pseudo-first-order reaction constant, by the second-order reaction constant for pure catalase ($k = 4.6 \times 10^7$ M⁻¹·s⁻¹; Sies *et al.*, 1973).

Superoxide dismutase activity was measured according to the assay described by McCord & Fridovich (1969) at pH 7.4. The assay was done in the absence and presence of 1 mM-KCN, and the measured activities are referred to as Cu-Zn-superoxide dismutase (cyanide-sensitive) and Mn-superoxide dismutase (cyanide-insensitive).

Oxygen uptake and H₂O₂ production by isolated brain mitochondria

The brains of four rats were pooled and homogenized at a concentration of 9 ml/g of tissue in a medium consisting of 0.23 M-mannitol, 0.07 M-sucrose, 1 mM-EDTA and 10 mM-Tris/HCl, pH 7.3. The homogenate was centrifuged at 600 *g* for 10 min, the pellet discarded and the supernatant centrifuged at 8000 *g* for 10 min. The pellet was washed twice with the same medium. All operations were performed at 0–2 °C.

Oxygen uptake was measured with a Clark electrode at 30 °C in a reaction medium consisting of 0.23 M-mannitol, 0.07 M-sucrose, 1 mM-EDTA, 5 mM-phosphate and 20 mM-Tris/HCl, pH 7.2.

H₂O₂ was assayed by the formation of cytochrome *c* peroxidase-H₂O₂ complex in a reaction medium consisting of 0.23 M-mannitol, 0.07 M-sucrose and 20 mM-Tris/HCl, pH 7.4. Cytochrome *c* peroxidase was added at a concentration of 0.5–1.0 μ M. The rate of formation of peroxidase-H₂O₂ was measured at 424–400 nm in a Perkin-Elmer 356 double-beam spectrophotometer at 30 °C (Boveris, 1984).

Chemicals

Tri-iodothyronine was provided by Glaxo (Buenos Aires, Argentina). Enzymes and chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistics

Values in Tables are means $\pm$ S.E.M., and *n* indicates the number of animals or independent assays in each group. Statistical analysis was performed using Student's *t* test.

RESULTS

Oxygen uptake by brain slices

The oxygen uptake by brain slices of neonatal hyperthyroid rats showed an increase of 30% over the corresponding value for control rats of the same age, which was 0.33 ± 0.3 μ mol O₂/min per g of brain. Hyperthyroid rats showed a dramatic decrease in lifespan; survival was shortened from about 100 weeks to 14 ± 2 weeks.

Organ and homogenate chemiluminescence of the brain

Brain chemiluminescence was measured *in situ* by using two surgical approaches. In one of them, the skin was removed and a circular window of about 2 cm

Table 1. Brain chemiluminescence of hyperthyroid rats

	Control (n = 6)	Hyperthyroid (n = 6)
Brain chemiluminescence <i>in situ</i> (c.p.s./cm ²)		
1. Exposed brain	130 ± 13	190 ± 30*
2. Through parietal bones	100 ± 10	170 ± 8*
Brain homogenate chemiluminescence (c.p.m./mg of protein)	7700 ± 530	6100 ± 600
*P < 0.001		

diameter was cut into the parietal bones and the brain exposed. In the other procedure, skin was removed and chemiluminescence was measured through the translucent parietal bones.

The spontaneous chemiluminescence of the exposed brain of control rats was 130 ± 12 c.p.s./cm². This value is higher than that (72 c.p.s./cm²) previously reported by Boveris *et al.* (1981) for adult rats. This phenomenon of higher values of organ chemiluminescence in young animals had also been noticed for the liver (Boveris *et al.*, 1983; Fraga *et al.*, 1987). The exposed brain of hyperthyroid rats showed an increase in emission of about 46% (Table 1). Spontaneous brain chemiluminescence measured through the translucent parietal bones and thus avoiding surgical trauma was 99 ± 10 c.p.s./cm²; the 30% decrease in brain emission is thought to be due to scattering of the emitted light by the parietal bones. In the hyperthyroid animals, brain chemiluminescence measured with this surgical approach was 70% higher than in the controls (Table 1). Brain emissions were stable for periods of 10–30 min and dead animals gave no chemiluminescence; these properties were taken as an indication that bone fluorescence was not involved.

The spontaneous chemiluminescence of diluted brain homogenates from control rats was 7700 ± 530 c.p.m./mg of protein and the brain homogenates of hyperthyroid rats showed a slight decrease in chemiluminescence which was not statistically significant (Table 1).

Activities of antioxidant enzymes

The activities of some of the main antioxidant enzymes of rat brain were increased by tri-iodothyronine treat-

Table 2. Activity of antioxidant enzymes in the brain of hyperthyroid rats

	Control (n = 8)	Hyperthyroid (n = 8)
Cu-Zn-superoxide dismutase (units/g of brain)	3520 ± 100	4150 ± 120*
Mn-superoxide dismutase (units/g of brain)	190 ± 10	210 ± 10
Catalase (nmol/g of brain)	0.11 ± 0.1	0.15 ± 0.1*
Se-glutathione peroxidase (munits/g of brain)	1.85 ± 0.11	2.41 ± 0.20*
Non-Se-glutathione peroxidase (munits/g of brain)	0.65 ± 0.09	0.74 ± 0.10
*P < 0.01		

Table 3. H₂O₂ production and oxygen uptake in isolated brain mitochondria

	Control (n = 4)	Hyperthyroid (n = 4)
H ₂ O ₂ production (nmol/min per mg of protein)		
State 4	0.20 ± 0.01	0.18 ± 0.01
State 3	0.02 ± 0.01	0.02 ± 0.01
O ₂ uptake (nmol/min per mg of protein)		
State 4	10.2 ± 0.8	9.4 ± 0.9
State 3	42 ± 4	40 ± 5
Isolated mitochondrial protein (g)	15.1 ± 1.4	14.8 ± 2.0

Fig. 2. H₂O₂ production by mitochondria isolated from the brain of young normal rats

The numbers near the traces indicate H₂O₂ production in nmol/min per mg of protein. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

ment. Cu-Zn-superoxide dismutase activity was increased by 18%, whereas the Mn-superoxide dismutase activity was not affected (Table 2). Catalase and Se-dependent glutathione peroxidase activities were increased by 36 and 20% respectively by hyperthyroidism, whereas non-Se-dependent glutathione peroxidase activity was not affected (Table 2).

H₂O₂ production and oxygen uptake by brain mitochondria

Brain mitochondria isolated from normal and hyperthyroid rats were assayed for H₂O₂ production and oxygen uptake (Table 3). H₂O₂ production and oxygen uptake were similar for both types of animals (Table 3). Fig. 2 illustrates the main features of H₂O₂ production by isolated rat brain mitochondria; a relatively high rate in state 4 that is markedly reduced in the transition to state 3 and increased to maximal values by addition of antimycin A.

DISCUSSION

Neonatal hyperthyroidism causes marked changes in whole brain metabolism (Walravens & Chase 1969), such as an increase in oxygen uptake and a reduction in

the number of oligodendroglia (Pelton & Bass, 1973). Thyroxine-treated animals initiate myelinogenesis earlier (Walters & Morell, 1981) but hyperthyroid animals show a pronounced myelin deficit at advanced age (Faryna de Raveglia *et al.*, 1973). The deficit may be interpreted as the consequence of oxidative stress in the hyperthyroid brain. Myelin is highly susceptible to the attack of reactive oxygen species as indicated by the peroxidation of the membrane lipids (Chan *et al.*, 1982), and oxidative damage to myelin has been proposed as a primary factor in the acceleration of ageing in the human brain (Chia *et al.*, 1983).

The oxygen uptake by brain slices of hyperthyroid rats indicates the existence of a hypermetabolic state. Most of the oxygen uptake of eukaryotic mammalian cells proceeds through the reaction of mitochondrial cytochrome oxidase coupled to energy (ATP)-yielding processes and produces H_2O_2 as the final product of oxygen reduction. However, an important fraction of oxygen uptake proceeds through univalent and bivalent reduction of oxygen yielding $\text{O}_2^{\cdot-}$ and H_2O_2 . This primary production of $\text{O}_2^{\cdot-}$ and H_2O_2 in the mitochondrial membranes seems to afford the most important physiological source of these oxygen species in eukaryotic cells relatively devoid of the microsomal electron transport chain, such as the brain cells (Chance *et al.*, 1979). The production of $\text{O}_2^{\cdot-}$ and H_2O_2 and the activity of the antioxidant enzymes determine intracellular steady-state levels of $\text{O}_2^{\cdot-}$ and H_2O_2 . These values have been calculated to be about 10^{-11} – 10^{-10} M- $\text{O}_2^{\cdot-}$ and 10^{-8} – 10^{-7} M- H_2O_2 for rat liver (Chance *et al.*, 1979).

A previous study (Loschen *et al.*, 1974) had failed to detect H_2O_2 generation by brain mitochondria although $\text{O}_2^{\cdot-}$ production had been reported (Forman & Kennedy, 1976). Our present results showing H_2O_2 production of 0.18–0.20 nmol/min per mg of protein in state 4 mitochondria with succinate as substrate in both normal and hyperthyroid brain mitochondria agree with the more recent report by Patole *et al.* (1986). Mitochondrial H_2O_2 will diffuse into the cytosol and to microperoxisomal catalase. On the basis of the rate of H_2O_2 production (about 0.5×10^{-7} M $^{-1}$ ·s $^{-1}$) and catalase content (0.1×10^{-6} M) of the brain, and by using the steady-state assumption where: $d[\text{H}_2\text{O}_2]/dt = -d[\text{H}_2\text{O}_2]/dt = k[\text{cat}][\text{H}_2\text{O}_2]$ and a value of $k = 4.6 \times 10^7$ M $^{-1}$ ·s $^{-1}$, a steady-state level of $[\text{H}_2\text{O}_2]$ of approx. 10^{-8} M can be estimated. This value may be taken as an overestimation since glutathione peroxidase activity was not considered.

The altered molecular mechanisms by which the hypermetabolic state of hyperthyroidism leads to oxidative stress remain to be explained. Given the similar mitochondrial mass (Table 3; Faryna de Raveglia *et al.*, 1982), the higher rate of oxygen uptake in hyperthyroidism will shift the mitochondrial metabolic state to a more active condition, i.e. closer to state 3, with a lower rate of H_2O_2 formation. However, the hypermetabolic state may also reduce some antioxidant factors such as NADPH and GSH levels, or increase the intracellular availability of a form of iron (ferritin-like) susceptible to redox cycling by $\text{O}_2^{\cdot-}$ and H_2O_2 to effectively catalyse the Haber–Weiss reaction.

The increase in superoxide dismutase, catalase and glutathione peroxidase activities affording an increased antioxidant protection can be assumed as an incomplete compensatory mechanism since the hyperthyroid state

leads to a marked increase in spontaneous brain chemiluminescence and to a shortening of life-span.

Chemiluminescence seems to afford an organ a non-invasive assay that through the measurement of light emission integratively measures the rate of formation of excited species, mostly singlet oxygen (Cadenas & Sies, 1984), which are an indirect measurements of the steady-state level of $\text{ROO}^{\cdot}$ radicals (Boveris *et al.*, 1980, 1981; Cadenas & Sies, 1984; Fraga *et al.*, 1987).

Accordingly, the marked increase in the spontaneous chemiluminescence of the hyperthyroid rat brain appears to indicate an increase in the steady-state level of oxyradicals. Increased tissue chemiluminescence has been associated with the development of cell injury and necrosis in rat liver after the oxidative stress produced by selenium and vitamin E deficiency (Fraga *et al.*, 1987).

It is worth noting that upon comparable oxidative stress (i.e. oxygenation of diluted tissue homogenates), the brain yields about eight times more malondialdehyde and chemiluminescence than does the liver (Cadenas *et al.*, 1981). The lack of a difference in chemiluminescence of brain homogenates between normal and hyperthyroid rats appears to indicate a dynamic or metabolic origin for the increased spontaneous chemiluminescence of the hyperthyroid brain.

Hypermetabolism seems to lead to increased spontaneous chemiluminescence and to oxidative stress in the brain. It is apparent that these conditions in various organs may contribute to the observed shortening of lifespan in these animals.

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