Brain chemiluminescence and oxidative stress in hyperthyroid rats

Ana M. ADAMO,* Susana F. LLESUY, Juana M. PASQUINI and Alberto BOVERIS
Departamento de Quimica Biologica-IQUIFIB, Facultad de Farmacia y Bioquimica, 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 translucid 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 peroxyl 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 translucid parietal bones after exposing the skull, or (2) from the exposed brain, after removing the meninges and the parietal...
in nmol of NADPH oxidized/min per g of tissue (Flohe & Gunzler, 1984).

Catalase activity was determined by measuring the absorbance decrease at 240 nm in a reaction medium containing 2 mM-H$_2$O$_2$, 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 H$_2$O$_2$ 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.

H$_2$O$_2$ was assayed by the formation of cytochrome c peroxidase–H$_2$O$_2$ 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 μM. The rate of formation of peroxidase–H$_2$O$_2$ 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 ± 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 μmol O$_2$/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

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**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.m./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-H$_2$O$_2$ as oxidant. When H$_2$O$_2$ was used as oxidant, 20 μM-sodium azide was added to inhibit catalase. The activity with H$_2$O$_2$ 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

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**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 chemiluminescence of the exposed brain after removing the parietal bones. The circle indicates the exposed area.

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**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.
Brain chemiluminescence, oxidative stress and hyperthyroidism

Table 1. Brain chemiluminescence of hyperthyroid rats

<table>
<thead>
<tr>
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<th>Control (n = 6)</th>
<th>Hyperthyroid (n = 6)</th>
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<tbody>
<tr>
<td>Brain chemiluminescence</td>
<td></td>
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<tr>
<td><em>in situ</em> (c.p.s./cm²)</td>
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<tr>
<td>1. Exposed brain</td>
<td>130 ± 13</td>
<td>190 ± 30*</td>
</tr>
<tr>
<td>2. Through parietal bones</td>
<td>100 ± 10</td>
<td>170 ± 8*</td>
</tr>
<tr>
<td>Brain homogenate</td>
<td>7700 ± 530</td>
<td>6100 ± 600</td>
</tr>
<tr>
<td>chemiluminescence (c.p.m./mg of protein)</td>
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*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 translucid 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 translucid 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 treatment. 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).

\[ \frac{\text{H}_2\text{O}_2 \text{ production}}{\text{H}_2\text{O}_2 \text{ uptake}} \]

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

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

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 H₂O₂ as the final product of oxygen reduction. However, an important fraction of oxygen uptake proceeds through univalent and bivalent reduction of oxygen yielding O₂⁻ and H₂O₂. This primary production of O₂⁻ and H₂O₂ 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 O₂⁻ and H₂O₂ and the activity of the antioxidant enzymes determine intracellular steady-state levels of O₂⁻ and H₂O₂. These values have been calculated to be about 10⁻¹¹·10⁻¹⁰ M⁻O₂⁻ and 10⁻⁸·10⁻⁷ M⁻H₂O₂ for rat liver (Chance et al., 1979).

A previous study (Loschen et al., 1974) had failed to detect H₂O₂ generation by brain mitochondria although O₂⁻ production had been reported (Forman & Kennedy, 1976). Our present results showing H₂O₂ 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 H₂O₂ will diffuse into the cytosol and to microperoxisomal catalse. On the basis of the rate of H₂O₂ production (about 0.5 × 10⁻³ m⁻¹·s⁻¹) and catalese content (0.1 × 10⁻⁸ m) of the brain, and by using the steady-state assumption where: d[H₂O₂]/dt = -d[H₂O₂]/dt = k[cat][H₂O₂] and a value of k = 4.6 × 10⁷ m⁻¹·s⁻¹, a steady-state level of [H₂O₂] 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 H₂O₂ 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 O₂⁻ and H₂O₂ to effectively catalyse the Haber–Weiss reaction.

The increase in superoxide dismutase, catalese 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 lifespan.

Chemiluminescence seems to afford an organ a non-invasive essay 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 ROO• 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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