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Hyperthyroidism modifies ecto-nucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats in different phases of development

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Abstract

Here we investigate the possible effects of the hyperthyroidism on the hydrolysis of the ATP to adenosine in the synaptosomes of hippocampus, cerebral cortex and blood serum of rats in different developmental phases. Manifestations of hyperthyroidism include anxiety, nervousness, tachycardia, physical hyperactivity and weight loss amongst others. The thyroid hormones modulate a number of physiological functions in central nervous system, including development, function, expression of adenosine A_1 receptors and transport of neuromodulator adenosine. Thus, hyperthyroidism was induced in male Wistar rats (5-, 60-, 150- and 330-day old) by daily injections of L-thyroxine (T4) for 14 days. Nucleotide hydrolysis was decreased by about 14–52% in both hippocampus and cerebral cortex in 5 to 60-day-old rats. These changes were also observed in rat blood serum. In addition, in 11-month-old rats, inhibition of ADP and AMP hydrolysis persisted in the hippocampus, whereas, in cerebral cortex, an increase in AMP hydrolysis was detected. Thus, hyperthyroidism affects the extracellular nucleotides balance and adenosine production, interfering in neurotransmitter release, development and others physiological processes in different systems.

Keywords: Hyperthyroidism; Adenosine; Nucleotidases; L-Thyroxine; Development; Nucleotide hydrolysis

1. Introduction

In addition to the systemic effects of thyroid hormones, many investigations have focused on the brain activity (Kastellakis and Valcana, 1989; Margarity and Valcana, 1999). The thyroid hormones are crucial determinants of normal development, especially in the central nervous system (CNS) (Oppenheimer and Schwartz, 1997). The relatively inactive thyroid hormone, thyroxine (T4), is converted to the more active form, triiodotyronine (T3), in brain tissue by the action of the enzyme deiodinase type II (Tanaka et al., 1981; Courtin et al., 1986). However, the mechanisms of the direct actions of thyroid hormones in the adult CNS are poorly understood. It has been demonstrated that all the isoforms of thyroid hormone are expressed in the CNS, and high levels of thyroid hormone receptors are found in different areas in the brain at both developmental and adult stages (Calzà et al., 1997).

Disorders involving thyroid hormones are common and might lead to irreversible changes in the chemical wiring of the central nervous system. The excess of thyroid hormone is associated with different actions in CNS. Hyperthyroidism is characterized by nervousness, anxiety, physical hyperactivity, weight loss, increased perspiration, increase in metabolic routes and, in the most severe situations, seizures (Orgiazzi and Mornex, 1990; Sarkar and Ray,

Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CNS, central nervous system; NTPDase, nucleotide triphosphate diphosphohydrolase; Pi, inorganic phosphate; TCA, trichloroacetic acid; TSH, thyroid-stimulating hormone; T4, thyroxine

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1994). Circumstantial evidence indicates that thyroid hormone treatment increases the synthesis and the sensibility of central catecholamine receptors in the adult brain (Engstron et al., 1974). Additionally, thyroid hormones may also modulate both adenosine transport and A_1 receptors in rat brain (Fideu et al., 1994).

Adenosine is involved in various physiological and pathological processes, both in the periphery and in the CNS (Palmer and Stiles, 1995; Dunwiddie and Masino, 2001). It is now well documented that adenosine plays an important regulatory role in the functioning, differentiation and survival of developing neural cells (Heilbronn et al., 1995).

The inhibitory actions of adenosine on neurotransmitter release are mediated by specific plasma membrane A_1 receptors (Dragunow, 1988; Gilman, 1987). In brain, the A_1 adenosine receptor is the most prevalent adenosine receptor subtype, presenting a widespread and high level of expression (Dunwiddie and Masino, 2001). Furthermore, adenosine plays a significant role in intracellular signal transduction mechanisms depressing the release of thyroid-stimulating hormone (TSH) by A_1 receptor activation (Kumari et al., 1999; Robberencht et al., 1979). Adenosine can reach the extracellular space of the brain by release from cells via adenosine transporters or by dephosphorylation of adenine nucleotides via ecto-nucleotidases. Previous studies have demonstrated that, in the central nervous system, the neurotransmitter ATP is hydrolysed to adenosine by the conjugated action of ecto-nucleotidases, which include an ATP diphosphohydrolase (NTPDase3, CD39, ecto-apyrase, EC 3.6.1.5) and a 5′-nucleotidase (lymphocyte surface protein, CD73, EC 3.1.3.5.) (Battasttini et al., 1991; Bonan et al., 1998).

Furthermore, soluble nucleotidases are also involved in ATP breakdown to adenosine (Todorov et al., 1997). The nucleotidases actions are important in the maintenance of the levels of extracellular nucleotides and nucleosides.

Thus, the effect of the thyroid hormones on nucleotidases (ecto and soluble) activities can influence the actions mediated by the adenine nucleotides and allowing us to understand the features involved in thyroid dysfunction.

Since the thyroid hormones are associated with an increase in the adenosine transport in brain and that adenosine plays an important modulatory role in physiological and pathological situations, the present study investigates the effect of hyperthyroidism upon the pathway of adenosine formation via hydrolysis of ATP, ADP and AMP in blood serum and synaptosomes of cortex and hippocampus of rats in different phases of development.

2. Experimental procedures

2.1. Materials

Thyroxine, nucleotides (ATP, ADP, AMP), Malachite Green Base, Coomassie Brilliant Blue G, HEPES, Trizma base, EDTA and Percoll were obtained from Sigma (St. Louis, MO, USA). Percoll was routinely filtered through Millipore AP15 prefilters to remove aggregated, incompletely coated particles. All other reagents were of the highest analytical grade.

2.2. Induction of hyperthyroidism

Male Wistar rats in the following developmental phases were used throughout this study: neonatal (5-day-old rats; weighting 6–10 g), prepubertal (30-day-old rats; weighting 100–150 g) and sexually mature adult (60-, 150- and 330-day-old rats; weighting 200–600 g). Animals were housed in cages with food and water available ad libitum and were maintained under a 12-h light:12-h dark cycle (light on at 07:00 a.m.) at room temperature of 25 °C. Hyperthyroidism was induced by daily intraperitoneal injections of L-thyroxine (T4), 25 μg/100 g body weight, for 14 days (Friberg et al., 1985; Pantos et al., 2000). T4 was dissolved using 0.04 M NaOH and the final solution was prepared with saline solution. Control animals received intraperitoneal injections of saline solution. This treatment results in a long-term moderate hyperthyroidism (Pantos et al., 2000). Animals were killed by decapitation 24 h after the last injection. Then, the animals were used aged 20-, 45-, 75-, 165- and 345-day old. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior (SBNeC).

2.3. Subcellular fractionation

The rats were killed by decapitation and their hippocampus and brain cortex were removed and gently homogenized in 5 vol. of an ice-cold medium consisting of 0.32 M sucrose, 0.0001 M EDTA and 0.005 M HEPES, pH 7.5, with a motor-driven Teflon-glass homogenizer. The synaptosomes from hippocampus were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of the crude mitochondrial fraction were mixed with 4.0 ml of 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were then washed twice at 12,000 × *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.4. Isolation of blood serum fraction

Blood was drawn after the decapitation of male Wistar rats of different ages. Blood samples were centrifuged in plastic tubes for 5 min at 5000 × *g* at 20 °C and the serum was maintained on ice at 4 °C throughout the experiments.

2.5. Enzyme assays

The reaction medium used to assay ATP and ADP hydrolysis in the synaptosomal preparation was essentially as described previously (Battasttini et al., 1991) and contained 0.005 M KCl, 0.0015 M $CaCl_2$, 0.0001 M EDTA, 0.01 M glucose, 0.225 M sucrose and 0.045 M Tris–HCl buffer, pH 8.0, in a final volume of 200 μl.

The reaction medium used to assay 5′-nucleotidase activity contained 0.01 M $MgCl_2$, 0.1 M Tris–HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984).

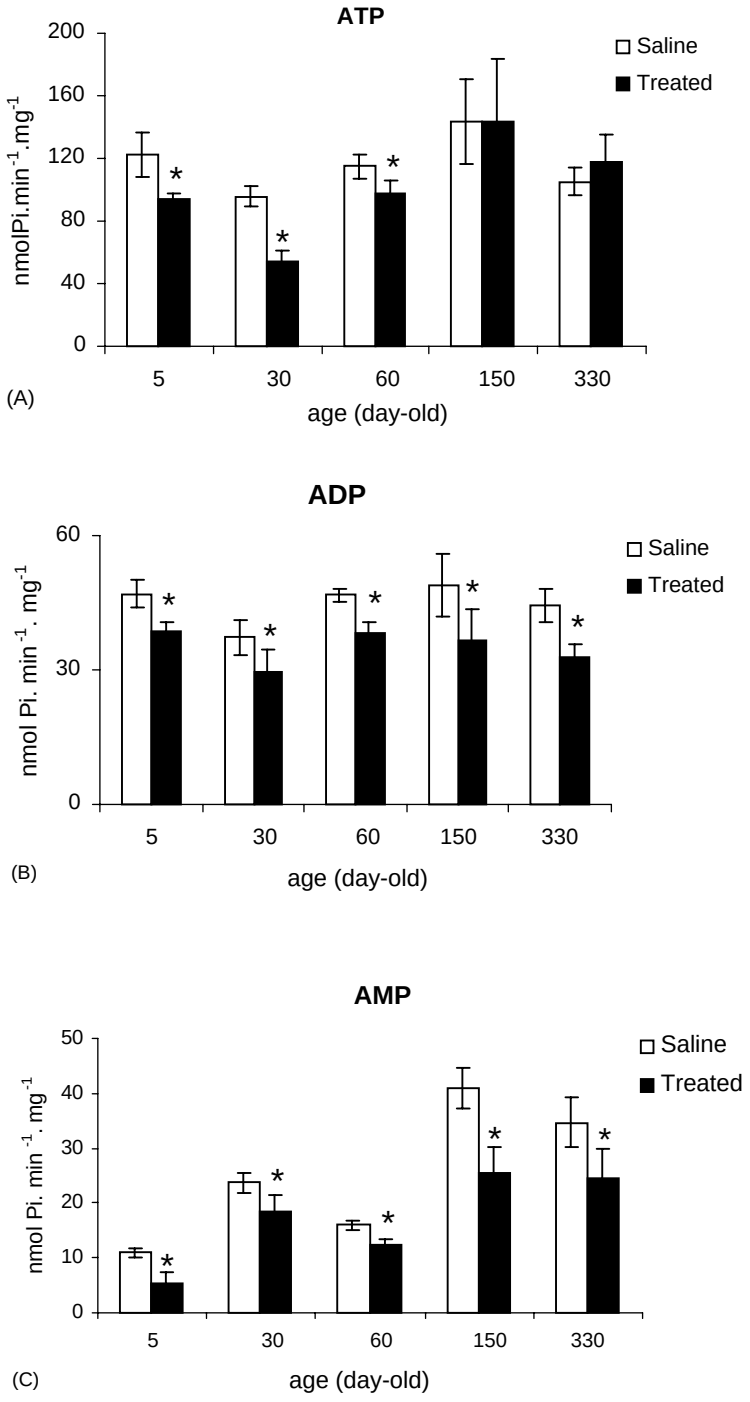

Fig. 1. Effects of hyperthyroidism induced by chronic administration of T4 (25 μg/100 g body weight, i.p.) on ATP (A); ADP (B) and AMP (C) hydrolysis in hippocampal synaptosomes of rats in different phases of development (5–330-day old). Bars represent means ± S.D. of at least five animals. The symbol (∗) indicates: T4-treated group significantly different from control group ($P < 0.05$, Student's *t*-test).

The synaptosomal fraction (10–20 μg protein) was added to the reaction mixture, pre-incubated for 10 min and incubated for 20 min at 37 °C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 0.001 M and stopped by the addition of 0.2 ml 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 μl samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986).

The enzymatic assays in rat blood serum were determined using the method described by Bruno et al. (2002).

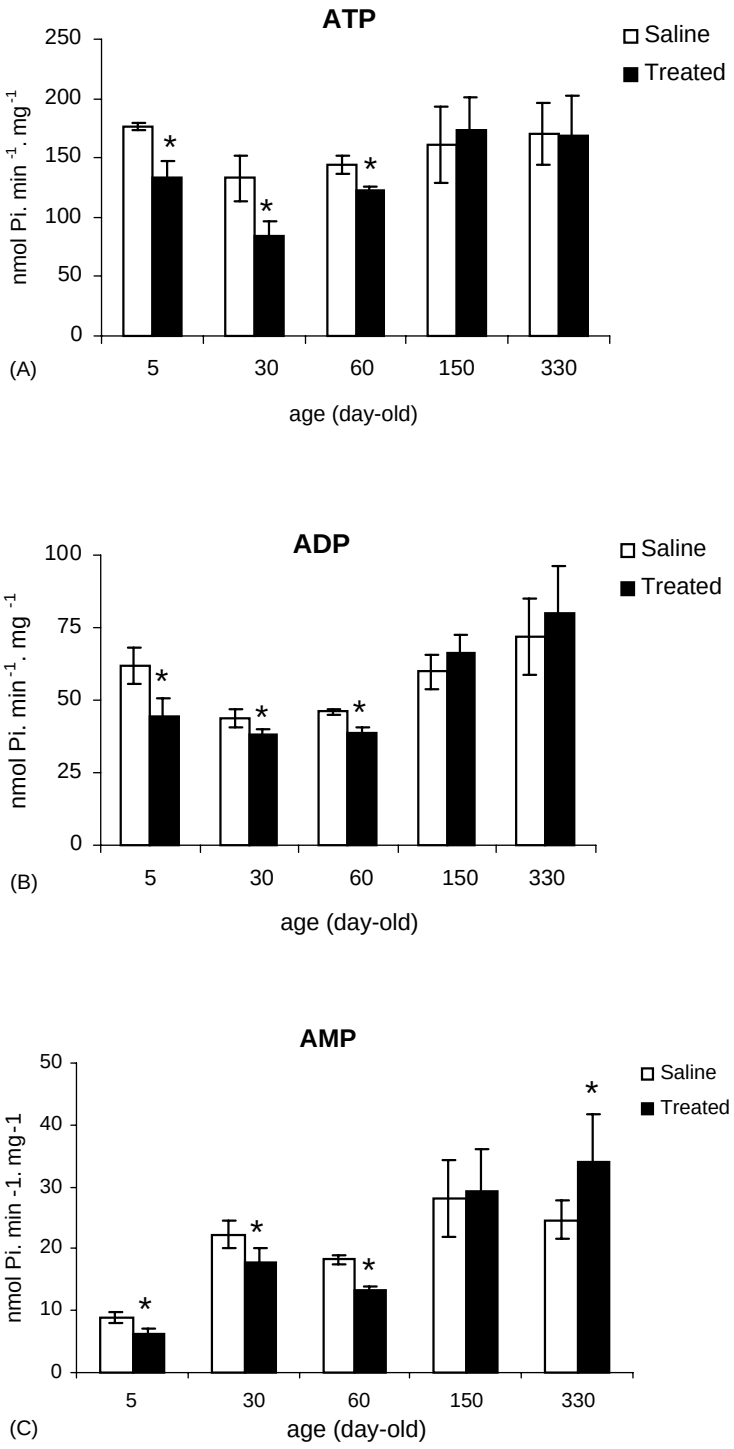

Fig. 2. Effects of hyperthyroidism induced by chronic administration of T4 (25 μg/100 g body weight, i.p.) on ATP (A); ADP (B) and AMP (C) hydrolysis in synaptosomes from cerebral cortex of rats in different phases of development (5–330-day-old). Bars represent means ± S.D. of at least five animals. The symbol (∗) indicates: T4-treated group significantly different from control group ($P < 0.05$, Student's *t*-test).

The samples were incubated for 40 min at 37 °C in a final volume of 0.2 ml containing serum protein in the range 1.0 mg to 1.5 mg, 0.1125 M Tris–HCl, pH 8.0 for ATP and ADP hydrolysis and 0.1 M Tris–HCl, pH 7.5 for AMP hydrolysis. The reaction was started by adding ATP, ADP or AMP to a final concentration of 0.003 M and stopped by the addition of 0.2 ml 10% trichloroacetic acid.

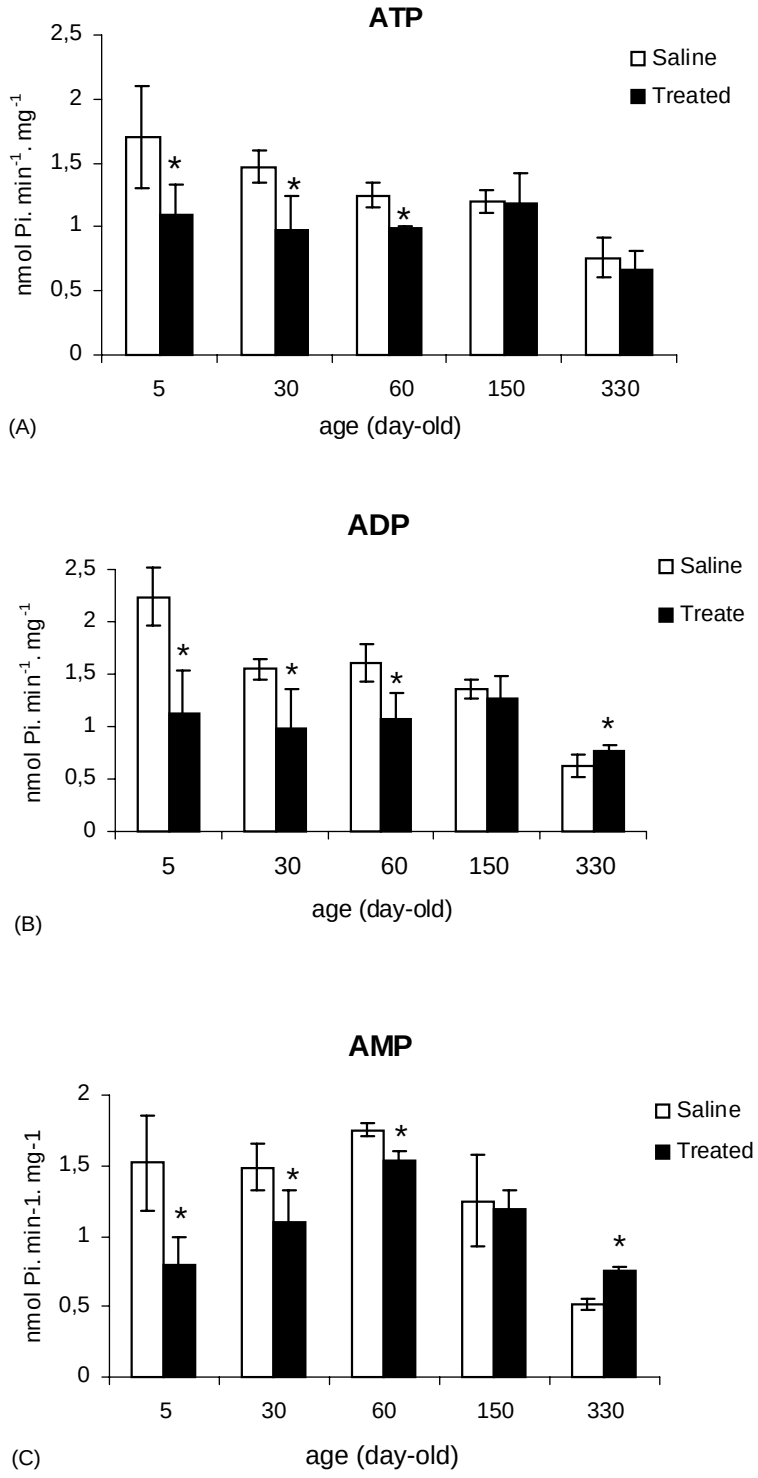

Fig. 3. Effects of hyperthyroidism induced by chronic administration of T4 (25 μg/100 g body weight, i.p.) on ATP (A); ADP (B) and AMP (C) hydrolysis in blood serum of rats in different phases of development (5–330-day old). Bars represent means ± S.D. of at least five animals. The symbol (∗) indicates: T4-treated group significantly different from control group ($P < 0.05$, Student's *t*-test).

For both assays, the incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown) and absorbance was measured at 630 nm. Inorganic phosphate released was determined as previously described by Chan et al. (1986). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid (TCA) were used to correct nonenzymatic hydrolysis of the substrates. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles of Pi released per minute per milligram of protein.

2.6. Protein determination

Protein was determined by the Coomassie Blue method, according to Bradford (1976) using bovine serum albumin as standard.

2.7. Statistical analysis

The data obtained are expressed as means ± S.D. values of at least five experiments. The results were analysed statistically by Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

The rats rendered hyperthyroid by treatment with thyroxine demonstrated symptoms such as hyperactivity and weight loss (data not shown). These effects were observed in all ages tested. In addition, the rats that initiated the treatment at postnatal day 5, demonstrated a faster development than control rats of the same age.

The effect of hyperthyroidism upon ATP, ADP and AMP hydrolysis in synaptosomal fractions from hippocampus, cortex and rat blood serum was evaluated.

Treatment with T4 caused a significant inhibition in ATP, ADP and AMP hydrolysis in hippocampal synaptosomes from animals of different ages (Fig. 1). In hippocampus from rats that started the treatment at postnatal day 5, we observed inhibitions of the 24, 18 and 52% in the hydrolysis of ATP (Fig. 1A), ADP (Fig. 1B) and AMP (Fig. 1C), respectively. In 30-day-old rats, the ATP (Fig. 1A), ADP (Fig. 1B) and AMP (Fig. 1C) hydrolysis, in hippocampal synaptosomes, was inhibited 44, 21 and 22%, respectively whereas in 60-day-old rats the hydrolysis of these nucleotides was inhibited 18, 22 and 27% in relation to control animals. In contrast, the hydrolysis of ATP in hippocampus from 150- and 330-day-old rats treated with T4, was not changed when compared to control rats (Fig. 1A). The ADP and AMP hydrolysis was significantly inhibited by 26 and 38% in 150-day-old rats and 27 and 30% in 330-day-old rats, respectively (Figs. 1B and C).

In cerebral cortex from 5-day-old rats, ATP (Fig. 2A), ADP (Fig. 2B) and AMP (Fig. 2C) hydrolysis was significantly inhibited by 25, 29 and 29%, respectively. Similarly, in 30-day-old treated rats, the inhibition in hydrolysis of these nucleotides was 37, 14 and 21%, respectively, in relation to respective control animals. Furthermore, in cerebral cortex from 60-day-old animals, a significant inhibition of 16, 17 and 27% was observed in the hydrolysis of ATP, ADP and AMP. In 150- and 330-day-old treated rats, the ATP and ADP hydrolysis were not altered by hyperthyroidism (Figs. 2A and B). Conversely, in 330-day-old rats, AMP hydrolysis was significantly increased (37%) in treated animals when compared to animals that received saline solution only (Fig. 2C).

Accordingly, similar results were obtained in rat blood serum at different ages tested. The hydrolysis of ATP (Fig. 3A), ADP (Fig. 3B) and AMP (Fig. 3C) were significantly inhibited in the rat blood serum that started the treatment at postnatal day 5 (36, 50 and 49%, respectively), in 30-day-old rats (34, 37 and 27%, respectively), and in 60-day-old rats (21, 34 and 13%, respectively). Nevertheless, no significant change was observed in the hydrolysis of the nucleotides in serum from 150-day-old rats (Fig. 3). In contrast, the hydrolysis of ADP and AMP was significantly increased (23 and 42%, respectively) in serum from 330-day-old rats treated with T4.

4. Discussion

Alterations in structure, function and behavior as a consequence of thyroid dysfunction, have highlighted the importance of these hormones, especially in central nervous system development and in the maintenance of neuronal system function throughout life (Smith et al., 2002).

The inhibition of ATP, ADP and AMP hydrolysis observed in 5-, 30- and 60-day-old rats after the hyperthyroidism induction, may prolong the effect of nucleotides at their respective receptors and/or modulate various processes in the central nervous system. Since ATP is recognized as an excitatory neurotransmitter in the central nervous system (Di Iorio et al., 1998) and a number of pathologies are associated with increased excitatory neurotransmission, the inhibition in ATP hydrolysis, observed herein, may have critical consequences. Large quantities of extracellular ATP cause cell death via activation of P2Z/$P2X_7$ receptors (Schulze-Lohoff et al., 1998; Harada et al., 2000). In contrast, several P2 receptor antagonists are known to prevent neurotransmitter release, excitotoxicity or cell death (Volonte and Merlo, 1996; Volonte et al., 1999). Besides, the expression of P2 receptor is enhanced during maturation (Amadio et al., 2002). Thus, the effects of ATP as a cell death mediator are more prominent in fully differentiated brain and the inhibition of hydrolysis of this nucleotide may be more dangerous in this development phase.

In mature brain, the adenosine A_1 receptors are among the most widely distributed Gi-coupled receptors, permitting adenosine to broadly influence neural function, modulate the release of neurotransmitters and confer protection against

seizures and ischemic damage (Reppert et al., 1991). As in the adult brain, expression of the A_1 receptor mRNA is remarkably widespread in late fetal life with high levels in regions that include cortex and hippocampus (Weaver, 1996). Therefore, adenosine via interaction with the A_1 receptor is particularly important during maturation and have numerous and diverse effects on brain development. Hence, in the brain of 5-day-old rats treated with T4, the changes observed in the ecto-5′-nucleotidase activity (the rate-limiting enzyme of extracellular adenosine formation), could explain the severity of thyroid diseases in the initial phases of development.

There is a general agreement that, during the postnatal development, the central nervous system of rats demonstrates pronounced changes in morphological, physiological and biochemical parameters. In this study, we found enzyme activities to be changed by hyperthyroidism in function of age and type of biological fraction studied. In synaptosomes from hippocampus, hyperthyroidism elicited a significant inhibition in ADP and AMP hydrolysis in rats of all ages tested, but the ATP hydrolysis was not modified in more mature rats (Fig. 1). In contrast, in the cerebral cortex of 330-day-old treated rats, AMP hydrolysis was activated (Fig. 2C).

Downregulation and reduced responsiveness of presynaptic adenosine A_1 receptors has been demonstrated in the central system nervous during aging, due to enhanced extracellular adenosine levels (Sperlágh et al., 1997). Because the excitatory A_{2A}-receptors are not altered during aging, the decrease in the A_1 receptors might shift the action of adenosine to the excitatory direction (Sperlágh et al., 1997). From this point of view, during aging, the importance of facilitatory A_{2A} receptors is greater and inhibitory A_1 receptors have a lower importance (Cunha et al., 2001). Thus, our findings in relation to differences obtained in nucleotide hydrolysis after induction of hyperthyroidism in later ages of development, could reflect a compensatory mechanism in response to the increase of the excitatory events found physiologically and in hyperthyroidism in these animals.

In addition, the inhibition of ATP and ADP hydrolysis, observed in blood serum from 5 to 60-day-old rats, can represent an increase in ATP-induced vasoconstriction (Konishi et al., 1999) and platelet aggregation mediated by ADP (Puri and Colman, 1997). A distinct behaviour of the enzymes was observed in the serum of rats in later phases of development in relation to younger rats (Fig. 3). The activation of hydrolysis of ADP and AMP observed in serum of 330-day-old rats treated with T4, suggest an increase in the levels of the potent vasodilator structure, adenosine (Fig. 3). These results obtained in serum, indicate the involvement of the soluble nucleotidases in hyperthyroidism and, possibly, effects mediated by ATP and ADP, such as platelet aggregation, may be attenuated in posterior phases of development (Puri and Colman, 1997). Several authors have described the important role of the ATP diphosphohydrolase, which maintains the physiological concentrations of the ADP in the process of haemostasis and thrombus formation (Frassetto et al., 1993; Marcus et al., 1997; Soslau and Youngprapakorn, 1997).

We recently demonstrated the effects, in vitro of the thyroid hormones upon the ectoenzymes activities in synaptosomes from rat brain. This study showed that T3 induces an inhibition of ATP and ADP hydrolysis. Conversely, T4 inhibited only the hydrolysis of ATP (Matos et al., 2002). Since, the AMP hydrolysis was not altered in vitro by theses hormones, it is reasonable to assume that the effects observed in hydrolysis of this nucleotide, in the present work, are in consequence of hyperthyroidism. In contrast to classic effects of the thyroid hormones that involve modulation of transcription of nuclear or mitochondrial genes, the in vitro effects of the thyroid hormones may be due to effects at the cell membrane level. However, the mechanisms by which these hormones may modulate the ecto-enzymes in the synaptosomal fraction require further study in future investigations.

In conclusion, hyperthyroidism affects both soluble and ecto-nucleotidases in different biological fractions and ages, consequently interfering in the balance of extracellular nucleotides and affecting the concerted function of the distinct physiological systems throughout the development. The presence of a shift in the balance of inhibitory and excitatory modulation after T4 treatment may be important for the understanding of the effects observed in hyperthyroidism.

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