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Nociceptive Response and Adenine Nucleotide Hydrolysis in Synaptosomes Isolated from Spinal Cord of Hypothyroid Rats

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Purinergic system exerts a significant influence on the modulation of pain pathways at the spinal site. Adenosine has antinociceptive properties in experimental and clinical situations, while ATP exerts pronociceptive actions in different pain models. In this study we investigated the hydrolysis of ATP to adenosine in synaptosomes from spinal cord in parallel with the nociceptive response of rats at different ages after hypothyroidism induction. Hypothyroidism elicited a significant increase in AMP hydrolysis to adenosine in synaptosomes from spinal cord of rats subjected to neonatal hypothyroidism and in 420-day-old rats submitted to thyroidectomy. Accordingly, these rats presented an analgesic response as a consequence of hypothyroidism. In contrast, the ATP hydrolysis was decreased in the spinal cord of 60-day-old hypothyroid rats in parallel with a significant increase in nociceptive response. These results indicate the involvement of adenine nucleotides in the control of the hypothyroidism-induced nociceptive response during development.

KEY WORDS: Adenosine; ATP; development; hypothyroidism; nociception.

INTRODUCTION

There is increasing evidence that adenosine and ATP may act as pain neuromodulators in spinal cord (1–3). ATP contributes to pain induction through its action on P2X receptors (4) and despolarization of spinal cord (5,6). In contrast, adenosine produces antinociception via activation of the adenosine A_1 receptors in spinal cord (7,8). This response results from the inhibition of adenylate cyclase, a decrease of cyclic AMP levels in the sensory nerve terminal inhibition of presynaptic voltage-sensitive Ca^{2+} channels and the activation of postsynaptic K^+ channels (8,9). Furthermore, the adenosine released in spinal cord contributes to the efficacy of opioid antinociception (2,10,11). On the basis on these findings, ectonucleotidases previously described in synaptosome isolated from spinal cord (12), which are responsible for the hydrolysis of the pronociceptive agent and excitatory neurotransmitter ATP to the neuromodulator and antinociceptive structure adenosine, are relevant to

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physiological pain control. In the central nervous system (CNS), ATP is hydrolyzed to adenosine by the conjugated action of an ecto-nucleotide triphosphate diphosphohydrolase family (NTPDases), responsible for ATP and ADP hydrolysis and a 5′-nucleotidase (CD73, EC 3.1.3.5) which hydrolyzes AMP formed to adenosine (13,14). Since these enzymes play a role in the availability of extracellular adenine nucleotide, their activities may be susceptive to hormonal and ontogenetic variations in different biological systems (12,15,16). We recently demonstrated changes in ATP hydrolysis to adenosine in brain synaptosomes of hyperthyroid rats in different developmental stages (16). Furthermore, we observed that the alterations in the nociceptive response of hyperthyroid rats are accompanied by changes in ectonucleotidases activities in synaptosomes from spinal cord at different ages (17). Other studies have also demonstrated alterations in extracellular adenosine levels in hyper- and hypothyroid states (18,19).

Disorders involving thyroid hormones are among the most common endocrine maladies and may lead to an extensive array of clinical manifestations. Hypothyroidism is characterized by memory impairment, menstrual irregularity, constipation, anemia, attentiveness, psychomotor deficits, psychiatric symptoms, somnolence and progressive intellectual deterioration (20). Furthermore, the severity of hypothyroidism symptoms is greatly influenced by the developmental stage studied (21).

Considering the effects that ATP and adenosine have on the nociception, the current study aims to investigate the ATP, ADP and AMP hydrolysis in synaptosomes from spinal cord and to compare these results with the nociceptive response obtained in hypothyroid rats at different developmental phases.

EXPERIMENTAL PROCEDURE

Materials

Methimazole, nucleotides (5′-ATP, 5′-ADP, 5′-AMP), Malachite Green Base, Coomassie Brilliant Blue G, HEPES, Trizma base, EDTA and Percoll were obtained from Sigma Chemical Co. (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.

Induction of Hypothyroidism

Male Wistar rats in the following developmental phases were used throughout this study: neonatal (14-days-old rats; weighting 24–26 g) and sexually mature adult (60 and 420-days-old rats; weighting 170–570 g).

For neonatal hypothyroidism induction, Wistar rats were mated and the day of appearance of the vaginal plug was considered as day 0 of fetal age. Neonatal hypothyroidism was induced by adding 0.02% methimazole in the drinking water during the whole day from day 9 of gestation, and throughout the whole of the experiment (22). Euthyroid rats with the same age were used as controls and submitted to the same environmental conditions as hypothyroid rats. Control and hypothyroid animals were decapitated at 14 days of age. These animals were obtained from five (for the nucleotide hydrolysis assays) or nine (for the tail-flick measurement) mothers of each group. Since this drug readily passes the placental barrier and is transmitted to the suckling pups in the mother's milk, the fetus and neonates also became profoundly hypothyroid (23). This protocol ensures a decreased growth rate and low levels of T3 and T4 (22).

Adult rats (60- and 420-day-old) were randomized sorted into three groups: control, sham-operated and hypothyroid. Hypothyroidism was induced by the surgical ablation of the thyroid gland (thyroidectomy) under ketamine and xylazine anesthesia. After the surgery, hypothyroid rats were treated with methimazole (0.05%) added to their drinking water during 14 days. The sham-operated group was also subjected to anesthesia and surgery as described above, but without ablation of the thyroid gland. The control, sham-operated and hypothyroid groups were killed by decapitation 15 days after the surgery.

All the animals were housed in cages with food and water available *ad libitum*. Animals were maintained under a 12-h light: 12-h dark cycle (light on at 07:00 a.m.) at a room temperature of 25°C. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior (SBNeC).

Subcellular Fractionation

The rats were killed by decapitation and the spinal cord was rapidly removed and gently homogenized in 10 volumes of an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5 with a motor-driven Teflon-glass homogenizer. Spinal cords obtained from 14-days-old rats were pooled to prepare the homogenates. The synaptosomes were isolated as described previously (24). Briefly, 0.5 ml of the crude mitochondrial fraction were mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10/20%). Percoll interface was collected with a wide-tip disposable plastic transfer pipette, centrifuged twice at 12,000 × *g* for 20 min and 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.

Enzyme Assays

The reaction medium used to evaluate ATP and ADP hydrolysis in the synaptosomal preparation was essentially as described previously (13) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl.

The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200 μl (25).

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 1.0 mM and was stopped by the addition of 0.2 ml 10% trichloroacetic acid (TCA). Samples were chilled on ice for 10 min and 100 μl aliquots were taken for the assay of released inorganic phosphate (Pi), which was determined as previously described by Chan et al. (26).

The time of incubation and protein concentration were chosen to ensure the linearity of the reaction (results not shown). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid 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 and absorbance was measured at 630 nm.

Protein Determination

Protein was determined by the Coomassie Blue method, according to Bradford (27) using bovine serum albumin as standard.

Tail-flick Measurement

Nociception was assessed with the tail-flick apparatus (28). The tail-flick model involves spinal nociceptive reflex, thus being suitable for the study of the adenosinergic influence in spinal cord (29). Rats were wrapped in a towel and placed on the apparatus. The light source (100 W) positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. Light intensity was adjusted so as to obtain a baseline tail-flick latency (TFL) of 3–6 s (s), using 0.5 mA. TFL represented the period of time (s) from the beginning of the trial to the tail deflection. A cut-off time of 10 s was used to prevent tissue damage. Animals were exposed to the tail-flick apparatus 24 h before the first measurement, to familiarize them with the procedure, since the novelty of the apparatus can itself induce antinociception (30).

Statistical Analysis

Data are expressed as means values ± S.D. values obtained from at least five animals per group for the adenine nucleotide hydrolysis experiments and at least nine animals per group to the results obtained for the tail-flick measurement. The results of neonatal hypothyroidism were analyzed by Student's *t* test. Data for the others ages were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's test. *P* values bellow 0.05 were considered significant.

RESULTS

Hypothyroidism was confirmed by measuring the hormones TSH, T3 and T4 in the blood serum of rats at the ages studied using chemoluminescent immunoassay (data not shown).

In order to exclude the effects of the thyroidectomy surgery in 60- and 420-day-old rats on the adenine nucleotide hydrolysis in spinal cord and nociceptive response, sham-operated rats were compared to control rats of the same age. In addition the effects of the hypothyroidism on the parameters analyzed, were verified by comparing 60- and 420-day-old rats submitted to hypothyroidism to sham-operated rats.

Fig. 1 demonstrates that the adenine nucleotide hydrolysis measured in synaptosomes from spinal cord from sham-operated rats was not significantly altered when compared to control animals at any age tested. This result excludes the influence of thyroidectomy surgery on ATP, ADP and AMP hydrolysis in 60- and 420-day-old rats.

Sham-operated rats were not processed for the 14-day-old rats since they were not submitted to surgery. As such, the data obtained for the 14-day-old rats were compared only to their respective control animals.

ATP and ADP hydrolysis were not altered in 14-day-old rats submitted to neonatal hypothyroidism (Fig. 1a, b) when compared to their respective control rats. However, the AMP hydrolysis (Fig. 1c) was significantly increased (35%) in rats 14-day-old rats submitted to neonatal hypothyroidism (15.97 ± 2.0 nmol Pi min^{-1} mg^{-1}, $P < 0.05$) in relation to control rats (11.86 ± 3.07 nmol Pi min^{-1} mg^{-1}). This result indicates a significant effect of congenital hypothyroidism upon the synaptosomal 5′-nucleotidase activity of the spinal cord (Fig. 1c).

With regard to the effects observed in 60-day-old rats, the ATP hydrolysis was inhibited by 15% in hypothyroid rats (146.02 ± 11.34 nmol Pi min^{-1} mg^{-1}, $P < 0.05$) in comparison to respective sham-operated rats (171.04 ± 16.06 nmol Pi min^{-1} mg^{-1}) (Fig. 1a). In contrast, ADP and AMP hydrolysis were not altered by hypothyroidism in this age (Fig. 1b, c).

In 420-day-old rats, ATP and ADP hydrolysis were not significantly changed by hypothyroidism in synaptosomes from spinal cord (Fig. 1a, b). Conversely, the AMP hydrolysis was increased by 42% in spinal cord from 420-day-old hypothyroid rats when compared to sham-operated animals (40.31 ± 5.18 vs. 28.27 ± 5.17 nmol Pi min^{-1} mg^{-1}, $P < 0.05$) (Fig. 1c).

The results presented in the Fig. 2 shows that the tail-flick latency measurement observed in sham-operated rats was not significantly different from control animals in any age tested, excluding the influence of thyroidectomy surgery on the nociceptive response in rats at different ages.

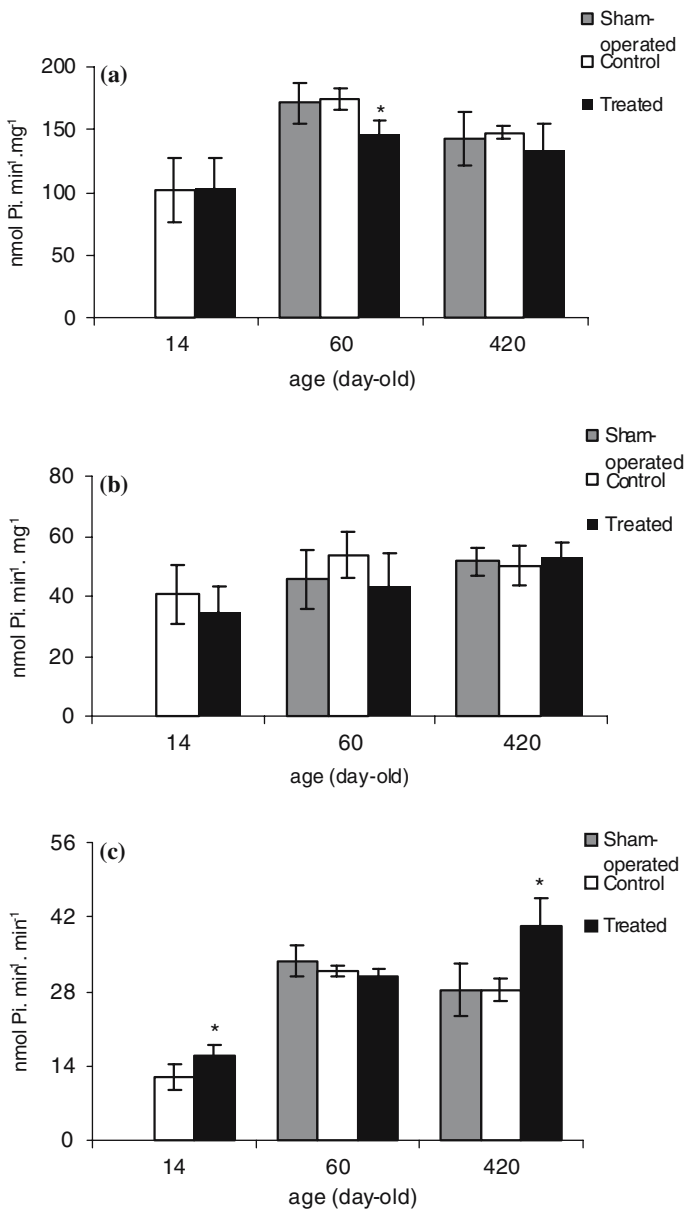

Fig. 1. Effects of hypothyroidism on ATP (a), ADP (b) and AMP (c) hydrolysis in synaptosomes from spinal cord in rats at 14-, 60- and 420-day-old. Bars represent means ±S.D. of at least five animals per group *Significantly different from control group to rats submitted to neonatal hypothyroidism (14-day-old rats obtained from five mothers of each group) ($P < 0.05$, Student's *t*-test) and significantly different from sham-operated group (60- and 420-day-old rats) ($P < 0.05$, one-way ANOVA followed by Duncan's test).

Hypothyroidism elicited a significant increase (55%) in tail-flick latency in 14-day-old rats submitted to neonatal hypothyroidism (4.71 ± 0.65 s, $P < 0.05$) in relation to control rats with the same age (3.03 ± 0.62 s) (Fig. 2). These results demonstrate that hypothyroidism decreases the pain threshold in 14-day-old rats, which coincides with the increase in AMP hydrolysis observed in rats at this age (Fig. 1c).

In contrast, 60-day-old hypothyroid rats showed a significant decrease of 35% in the tail-flick latency (2.7 ± 0.30 s, $P < 0.05$) in comparison to sham-operated rats (4.12 ± 0.62 s), indicating that hypothyroid rats are hyperalgesic at this developmental stage.

In addition, the tail-flick latency observed in 420-day-old rats was significantly increased by 41% in hypothyroid rats (5.03 ± 0.52 s, $P < 0.05$) when

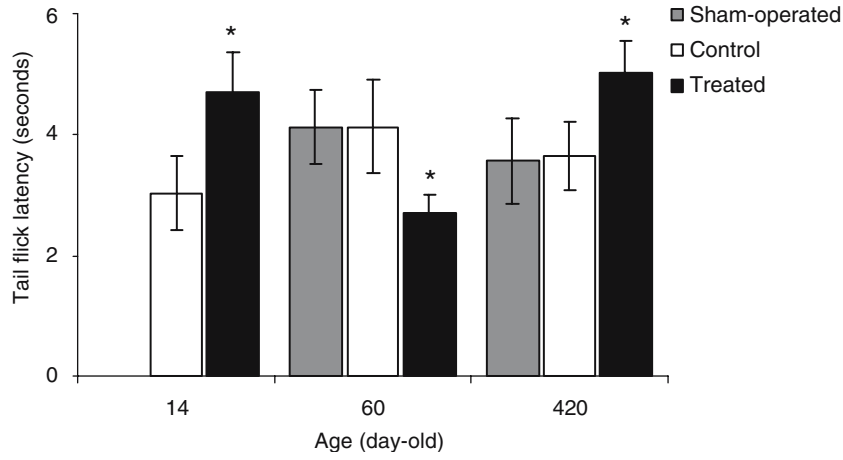

Fig. 2. Effects of hypothyroidism on the nociceptive response (verified by the tail-flick test) in rats at 14-, 60- and 420-day-old. Bars represent means ± S.D. of at least nine animals per group. *Significantly different from control group to rats submitted to neonatal hypothyroidism (14-day-old rats obtained from nine mothers of each group) ($P < 0.05$, Student's *t*-test) and significantly different from sham-operated group (60- and 420-day-old rats) ($P < 0.05$, one-way ANOVA followed by Duncan's test).

compared to the respective sham-operated rats (3.57 ± 0.70 s), characterizing an analgesic response induced by hypothyroidism in this age. Interestingly, these results coincide with the increase of 42% obtained for AMP hydrolysis in spinal cord from 420-day-old hypothyroid rats (Fig. 1c).

DISCUSSION

The influence of developmental stage on the biological processes that involve hormonal and enzymatic regulation is well documented. Thyroid hormones mediate important physiological effects in different biological systems throughout life. Hypothyroidism during a critical period of development has been associated with irreversible mental retardation and profound neurological deficits (20,31). In addition, both neurological and behavioral abnormalities are common in adult thyroid dysfunction (32).

Accordingly, in the present study the effects obtained after induction of hypothyroidism were dependent of the developmental stage studied.

In animals subjected to congenital hypothyroidism, the activity of 5′-nucleotidase, responsible for AMP hydrolysis to adenosine, was increased when compared to control rats. Moreover, this result was concomitant with decreased nociceptive response in these rats. Since adenosine released in the spinal cord has antinociceptive properties (8), the increase of adenosine production recorded herein may be associated with the inhibition of the nociceptive response of these animals. Furthermore, as these changes were observed in rats submitted to congenital hypothyroidism, the development of a variety of biochemical events may also have been affected by thyroid hormones deficiency during fetal and neonatal periods. Thyroid hormones deficiency in fetal and neonatal periods produces deleterious effects such as reduced synaptic connectivity, decreased myelination and alteration in the neurotransmitters levels (23,31,33). Thus, changes in adenosine production can be associated with some alterations noticed in neonatal hypothyroidism, since adenosine inhibits the neuronal excitability and the release of neurotransmitters via A_1-adenosine receptors (34), which are expressed in fetal rat spinal cord (35). Accordingly, the increase in adenosine levels in synaptosomes from spinal cord may activate the A_1-receptors and reduce the events related to neurotransmission leading to analgesia observed in hypothyroid rats.

The mechanism implicated in spinal antinociception includes a postsynaptic hyperpolarization of neuronal transmission by interactions with ATP-sensitive-K^+ channels (36). This action most likely accounts for inhibition of the release of substance P and excitatory amino acids in spinal cord (37,38), which may be mediated by adenosine analogs (8). The involvement of the purinergic system in the context of pain pathways formation also includes the neurotransmitter ATP (4), once that spinal cord neurons can be depolarized by this nucleotide (5) inducing the pain sensation (39).

In contrast to rats submitted to neonatal hypothyroidism, 60-days-old thyroidectomized rats showed a hyperalgesic response in the tail-flick test.

Concomitantly, the ATP hydrolysis was significantly inhibited in these rats. Hence, an increased availability of ATP as a pronociceptive agent in spinal cord nerve endings may well explain the hyperalgesic response observed in 60-day-old hypothyroid rats. This result also suggests that the thyroid hormones deficiency may affect the activity of an NTPDase2 (ecto-ATPase) in the spinal cord, and thus influence the processes modulated by ATP in this biological fraction.

With regard to the 420-day-old rats submitted to hypothyroidism, an analgesic response was observed accompanied by a significant activation of the synaptosomal 5′-nucleotidase activity in the spinal cord. Once again, a possible increase in the adenosine levels could justify the analgesic response observed in these rats.

The changes observed in both the hydrolysis of adenine nucleotide and the nociceptive response after hypothyroidism at different ages are expected, since the thyroid hormones effects are dependent upon developmental stage. Furthermore, the normal activities of adenine nucleotide hydrolysis-related enzymes are also altered during development (40,41). Other studies have shown that these enzymes can respond differently to physiological variations depending on the age studied (42), including the excess of thyroid hormones (16). Moreover, the receptors of ATP (P2) and adenosine (A_1 and A_2) present in the central nervous system are also modified as a function of age (35,43,44). Concerning to nociception, alterations in the concentration of adenine nucleotides and in the receptors that mediate their actions, also represent changes in the processes involved in the nociceptive pathway during development.

In summary, this study establishes a relationship between the adenine nucleotide hydrolysis in synaptosomes from spinal cord and the nociceptive response in hypothyroidism. Furthermore, the different results observed at different ages highlight the importance of developmental stage to this kind of endocrine disorders. In addition, the profile of purinergic agents and their analogs suggests a potential application of these agents for the development of novel analgesics that respond to this thyroid dysfunction.

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