Hyperthyroidism changes nociceptive response and ecto-nucleotidase activities in synaptosomes from spinal cord of rats in different phases of development

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Abstract

Changes in transport, receptors and production of extracellular adenosine have been observed after induction of hyperthyroidism. Adenosine is associated with inhibitory actions such as reduction in release of excitatory neurotransmitters and antinociception at spinal site. In contrast, ATP acts as an excitatory neurotransmitter and produces pronociceptive actions. ATP may be completely hydrolyzed to adenosine by an enzyme chain constituted by an ATP diphosphohydrolase and an ecto-5'-nucleotidase, as previously described in the spinal cord. Thus, we now investigated the effects of the hyperthyroidism on adenine nucleotide hydrolysis in the spinal cord and verified the nociceptive response in this pathology during different phases of development. Hyperthyroidism was induced in male Wistar rats, aged 5, 60 and 330 days by daily intraperitoneal injections of l-thyroxine (T4) for 14 days. Nociception was assessed with a tail-flick apparatus. Rats starting the treatment aged 5 days demonstrated a significant increase in ADP and AMP hydrolysis and increased tail-flick latency (TFL). In contrast, in the spinal cord from hyperthyroid rats aged 60 and 330 days old, the hydrolysis of ATP, ADP and AMP were significantly decreased. Accordingly, the tail-flick latency was decreased, indicating a hyperalgesic response. These results suggest the involvement of ecto-nucleotidases in the control of the hyperthyroidism-induced nociceptive response in rats at distinct developmental stages.

1. Introduction

Adenine nucleotides are known to be released in the extracellular space, where they act as important molecules for signaling (Vizi and Sperlagh, 1999). Extracellular ATP induces various biological responses, such as neurotransmission in both the peripheral and central nervous systems (Cunha and Ribeiro, 2000), excitability of spinal cord neurons (Sun et al., 1992), cell death via activation of P2Z/P2X7 receptors (Schulze-Lohoff et al., 1998; Harada et al., 2000) and activation of pain pathways in the spinal cord (Tsuda et al., 1999). It has been demonstrated that extracellular ATP is hydrolyzed by an ATP diphosphohydrolase (apyrase, NTPDase, EC 3.6.1.5) in synaptosomes of the peripheral and central nervous systems (Battistini et al., 1991; Sarkis and Saltó, 1991) generating AMP, which is...
later hydrolyzed by an ecto-5′-nucleotidase (EC 3.1.3.5) producing adenosine in the synaptic cleft. Alterations in ATP diphosphohydrolase and 5′-nucleotidase activity appear to be related to diverse situations such as epilepsy in brain synaptosomes and chronic stress in the spinal cord (Bonan et al., 2000; Torres et al., 2002). These enzymes control the availability of extracellular nucleotides, the interaction of nucleotides at their respective receptors and, consequently, the biological effects mediated by this interaction (Chen and Guidotti, 2001).

Adenosine acts as a neuromodulator primarily though inhibition of excitatory neurotransmitter release (Dunwiddie and Masino, 2001). The inhibitory actions of adenosine are mainly mediated by activation of A1 adenosine receptors (Fredholm and Dunwiddie, 1988), which are present in spinal cord (Choct et al., 1987). It has been proposed that adenosine is involved in physiological pain control at the spinal cord level and contributes to the action of opioid antinociception (Sollevi, 1997; Sweeney et al., 1989). In addition, adenosine may play a role in the pathophysiology of neuropathic pain, where pain-signaling mechanisms are altered (Guieu et al., 1996). The responses of adenosine receptors in relation to pain control are mediated by changes in cyclic AMP levels, inhibition of presynaptic voltage-gated calcium channel and via the activation of postsynaptic potassium channels (Salter et al., 1993; Sawynok, 1998). These results suggest a role of adenosine receptors in the modulation of both acute and chronic pain.

Furthermore, the extracellular levels of adenosine can be altered by a diverse array of pathological and physiological stimuli (Dunwiddie and Masino, 2001). Previous studies demonstrated that the thyroid hormones may modulate both nucleoside transporters and adenosine receptors of the A1 subtype in the central nervous system (Fideu et al., 1994). In addition to their well-established role in cellular metabolism, thyroid hormones have critical effects upon cellular differentiation, growth, sensibility and role in cellular metabolism, thyroid hormones have critical pathways in the spinal cord, the present study is aimed at investigating the effects of hyperthyroidism on the pain threshold and correlate the nociceptive mechanisms with the adenosine nucleotide hydrolysis in spinal cord during different phases of development.

2. Materials and methods

2.1. Induction of hyperthyroidism

Male Wistar rats (Rattus norvegicus), weighing 6–7 g (5-day-old rats), 220–300 g (60-day-old rats) and 420–580 g (330-day-old rats) were used throughout this study. 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 a room temperature of 25 °C. Hyperthyroidism was induced by daily intraperitoneal injections of t-thyroxine (T4), 25 μg/100 g body mass, for 14 days (Friberg et al., 1985). T4 was dissolved using 0.04 M NaOH, and the final solution was prepared with 0.9% saline solution. Control animals received intraperitoneal injections of 0.9% saline solution. Animals were killed by decapitation 24 h after the last injection. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Society for Neuroscience and Behavior (SBNeC).

2.2. 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. The synaptosomes 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 an 8.5%. Percoll solution was layered onto an isosmotic Percoll/sucrose discontinuous gradient (10%/20%). The Percoll interface was collected with a wide-tip disposable plastic transfer pipette, 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.

2.3. Enzyme assays

The reaction medium used to evaluate ATP and ADP hydrolysis in the synaptosomal preparation was essentially as described previously (Battasttini et al., 1991) and contained 5.0 mM KCl, 1.5 mM CaCl2, 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 ecto-5′-nucleotidase activity contained 10 mM MgCl2, 100 mM Tris–HCl, pH
7.5 and 0.15 M sucrose in a final volume of 200 μL (Heymann et al., 1984).

The synaptosomal fraction (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM, incubated for 20 min at 37 °C and was stopped by the addition of 0.2 mL 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min, and 100-μL samples were taken for the assay of released inorganic phosphate (Pi). Inorganic phosphate (Pi) released was determined as previously described by Chan et al. (1986).

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.

2.4. Protein determination

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

2.5. Tail-flick measurement

Nociception was assessed with the tail-flick apparatus (D’Amour and Smith, 1941). The tail-flick model involves spinal nociceptive reflex, being thus suitable to the study of adenosinergic influence in spinal cord (Keil and DeLander, 1994). Rats were wrapped in a towel and placed on the apparatus. The light source 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. The tail-flick latency (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. At the end of T4 treatment and 24 h before the first measurement, the animals were exposed to the tail-flick apparatus to familiarize them with the procedure since the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

2.6. Statistical analysis

The data obtained in the nucleotide hydrolysis experiments are expressed as means±S.D. values of at least five experiments. The results were analyzed statistically by Student’s t-test test. The results to the tail-flick are expressed as median (interquartile range) of eight animals and were analyzed by Mann–Whitney U-test (two-tailed). Values of P<0.05 were considered significant.

3. Results

Fig. 1 demonstrates the adenine nucleotide hydrolysis in spinal cord from hyperthyroid rats at ages tested. The ATP hydrolysis was not altered in rats that started the treatment at 5 days old (Fig. 1A) when compared to the control group. In contrast, the ADP hydrolysis was significantly increased (22%) in rats at the same age submitted to hyperthyroidism (56.2±3.4 nmol Pi min⁻¹ mg⁻¹, P<0.05) in relation to control rats (43.8±3 nmol Pi min⁻¹ mg⁻¹) (Fig. 1B). Moreover, a significant increase of 61% was observed in the AMP hydrolysis in treated rats (26.3±4.4 nmol Pi min⁻¹ mg⁻¹, P<0.05) at 5 days old in comparison to control rats (16.2±4.7 nmol Pi min⁻¹ mg⁻¹), indicating a significant
effect of hyperthyroidism on ecto-5'-nucleotidase activity of spinal cord nerve terminals (Fig. 1C).

In contrast to the effects observed in 5-day-old rats, hyperthyroidism elicited a significant decrease in the hydrolysis of ATP, ADP and AMP in spinal cord nerve terminals from 60-day-old rats. ATP hydrolysis was inhibited by 26% in treated rats (147.1±28 nmol Pi min⁻¹ mg⁻¹, P<0.05) in relation to control animals (200±13.6 nmol Pi min⁻¹ mg⁻¹; Fig. 1A). Similarly, a significant decrease of 25% in ADP hydrolysis was observed in rats submitted to treatment with T4 (40±3.7 nmol Pi min⁻¹ mg⁻¹, P<0.05) when compared to control rats (53.8±7.5 nmol Pi min⁻¹ mg⁻¹; Fig. 1B). The results for ecto-5'-nucleotidase activity showed a similar profile when compared to ATP and ADP hydrolysis. AMP hydrolysis was significantly inhibited (38%) in treated animals (19.9±3.4 nmol Pi min⁻¹ mg⁻¹, P<0.05) in relation to the animals that only received saline solution (31.9±2.5 nmol Pi min⁻¹ mg⁻¹; Fig. 1C).

In 330-day-old rats, ATP hydrolysis was significantly decreased by 21% in T4-treated rats (170.8±13 nmol Pi min⁻¹ mg⁻¹, P<0.05) in relation to control animals (214±21.8 nmol Pi min⁻¹ mg⁻¹) (Fig. 1A). Similarly, ADP hydrolysis in spinal cord nerve terminals was inhibited by 30% in treated rats (24.5±3.7 nmol Pi min⁻¹ mg⁻¹, P<0.05) (Fig. 1B) when compared to control rats (34.6±4.4 nmol Pi min⁻¹ mg⁻¹, P<0.05), whereas the decrease observed in AMP hydrolysis was 25% in treated rats (17.5±2 nmol Pi min⁻¹ mg⁻¹, P<0.05) in relation to control rats (23.3±3.1 nmol Pi min⁻¹ mg⁻¹; Fig. 1C).

To investigate the effects of hyperthyroidism on nociception in rats at different phases of the development, animals aged 5, 60 and 330 days were submitted to tail-flick latency test (Fig. 2). Hyperthyroidism elicited a significant increase in tail-flick latency in 5-day-old rats (7.9±1.2 s, P<0.05) in relation to control rats with the same age (3.8±0.2 s). These results indicate that hyperthyroidism decreases the pain threshold in 5-day-old rats, which coincides with the increase in ADP and AMP hydrolysis observed in rats at this age.

In contrast, 60-day-old treated rats showed a significant decrease in the tail-flick latency (2.34±0.8 s, P<0.05) in comparison to control animals (5±1 s), demonstrating that treatment with T4 increases the response to pain in rats at this developmental stage. The tail-flick latency observed in 330-day-old rats was significantly decreased in treated rats (2.6±0.7 s, P<0.05) when compared to their respective animals control (3.82±0.8 s), indicating that hyperthyroidism increased the nociceptive response in 330-day-old rats. Curiously, these results correspond to results obtained for adenine nucleotide hydrolysis, which was inhibited in these rats.

4. Discussion

Our results demonstrate modifications induced by hyperthyroidism in both nucleotide hydrolysis and nociception during different phases of development. In addition, these changes depended on the age of the studied animal and are in agreement with previous reports in the literature demonstrating that the effects of thyroid hormones, and consequently the alterations involving these hormones, are dependent on the developmental stage (Smith et al., 2002). Furthermore, the effects on the ecto-nucleotidases studied herein were also influenced by the age of the animal. During the postnatal development of the rat, the central nervous system exhibits modifications in a variety of biological processes, including biochemical events, as well as in the enzymes involved in neurotransmitter metabolism (Morgane et al., 1978). It has been shown that the effects observed in ATP diphosphohydrolase activity may vary depending on the age of the animals (Muller et al., 1990; Bruno et al., 2002). These studies suggested an important functional involvement of an ATP diphosphohydrolase during development. In addition, the activity of ecto-5'-nucleotidase, the rate-limiting enzyme in extracellular adenosine formation, is greatly modified depending on the age of the animals (Smith et al., 1980). Furthermore, thyroid hormones elicit a pronounced effect on the ontogeny of ecto-5'-nucleotidase, which shows a marked sensitivity to both hyper- and hypothyroidism (Smith et al., 1980).

In the present study, we observed a significant increase in ADP and AMP hydrolysis in the spinal cord from rats that started the treatment at postnatal day 5 (Fig. 1B; C). Since ADP is considered to be a substrate marker for ATP diphosphohydrolase activity (Battastini et al., 1991), this result suggests that hyperthyroidism can modulate the activity of this enzyme in spinal cord. The dissociation of the changes of ATP and ADP hydrolysis in 5-day-old rats may signify a distinct behavior of the ecto-ATP diphosphohydrolase regarding the two substrates in response to hyperthyroidism or could be explained by the coexistence
of this enzyme together with an ecto-ATPase (Kegel et al., 1997). The increase in AMP hydrolysis indicates the activation of ecto-5'-nucleotidase and consequently a possible enhancement in adenosine production elicited by the hyperthyroidism at this developmental stage. Since adenosine exerts antinociceptive actions in the spinal cord (Keil and DeLander, 1994; Sawynok, 1998), the decrease in pain sensitivity observed in 5-day-old treated rats (Fig. 2) might be related to the increase in ecto-5'-nucleotidase activity in this preparation. This inhibitory action of the adenosine in nociception appears to be mediated by the activation of the adenosine A1 receptors at a spinal site (Sawynok et al., 1986). Therefore, the analgesic effects observed after hyperthyroidism might involve the activation of A1 receptors by adenosine produced via AMP hydrolysis in the spinal cord of hyperthyroid rats. Moreover, a previous study demonstrated that the administration of an adenosine transport inhibitor induced an antinociceptive effect in naive rats, empathizing the importance of analgesic effect of adenosine produced via 5'-nucleotidase (Torres et al., 2003).

In addition to its antinociceptive effects, adenosine also plays a crucial role in survival and differentiation of developing neural cells (Heilbronn et al., 1995). For this reason, the production of adenosine might be important in animals during the initial phases of development and might be a beneficial mechanism to avoid the damage induced by cellular hyperexcitability caused by hyperthyroidism.

Interestingly, 60- and 330-day-old rats showed a sensitivity to thyroid hormones different from 5-day-old rats with regard to nucleotide hydrolysis and pain threshold. In 60- and 330-day-old rats, hyperthyroidism significantly decreased the hydrolysis of ATP, ADP and AMP (Fig. 1). Accordantly, hyperthyroid rats 60 and 330 days old exhibited a decreased tail-flick latency (Fig. 2), characteristic of hyperalgesic response. Considering the proalgesic properties of ATP and the involvement of the spinal cord in nociceptive processes, the inhibition of the hydrolysis of this nucleotide in the spinal cord from rats at these ages may be associated with the hyperalgesic response observed in the nociception test. In fact, both sensory neurons, as well as spinal cord neurons, can be depolarised by ATP (Jahr and Jessel, 1985). Thus, ATP acts in the context of pain pathways as an activator of nociceptive sensory neurons via ATP-gated ion channels, inducing the pain sensation (Bleehen and Keele, 1997).

Likewise, the inhibition of AMP hydrolysis, observed in 60- and 330-day-old rats, (Fig. 1C) confirms the thyroid-sensitive nature of ecto-5'-nucleotidase first observed in the heart (Smith et al., 1978), which might contribute to a decrease of adenosine levels. Considering the antinociceptive properties of adenosine, the hyperalgesic response observed in hyperthyroid 60- and 330-day-old rats (Fig. 2) may be associated with the inhibition of the ecto-5'-nucleotidase induced by hyperthyroidism in the spinal cord. Moreover, changes in adenosine levels are important not only from the point of view of nociception. Inhibition of Ca2+ entry into nerve terminals, mediated by adenosine, inhibits the release of excitatory amino acids (Dunwiddie and Masino, 2001). Thus, an inhibition of ecto-5'-nucleotidase activity in hyperthyroidism may affect the balance between excitatory and inhibitory transmission modulated by adenosine in the spinal cord.

In summary, the results of this study show that hyperthyroidism alters the adenine nucleotide hydrolysis in the spinal cord and that these changes coincide with the nociceptive response observed in the treated rats. These parallel findings suggest the involvement of adenine nucleotide hydrolysis-related enzymes in nociceptive pathways. Furthermore, it is important to observe that the changes in both nucleotide hydrolysis and nociceptive response were dependent of the developmental stage, confirming that the effects of the thyroid hormones vary with the ontogenetic period.

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