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Research Report

Intrastriatal injection of hypoxanthine alters striatal ectonucleotidase activities: A time-dependent effect

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ABSTRACT

The aim of this study was to investigate the effects of intrastriatal injection of hypoxanthine on ectonucleotidase (E-NTPDases and ecto-5′-nucleotidase) activities and expressions in the striatum of rats. The effect of pre-treatment with vitamins E and C on the effects elicited by this oxypurine on enzymatic activities and on thiobarbituric reactive substances (TBARS) was also investigated. The effect of pre-incubation with hypoxanthine on nucleotide hydrolysis in striatum homogenate was also determined. Adult Wistar rats were divided into (1) control and (2) hypoxanthine-injected groups. For ectonucleotidase activity determination, the animals were sacrificed at 30 min, 24 h and 7 days after drug infusion. For the evaluation of the expression of NTPDase 1–3 and also ecto-5′-nucleotidase, TBARS assay and the influence of the pre-treatment with vitamins on ectonucleotidase activities, the animals were sacrificed 24 h after hypoxanthine infusion. Results show that hypoxanthine infusion significantly inhibited ectonucleotidase activities and increased TBARS only 24 h after administration. Pre-treatment with vitamins was able to prevent these effects. Moreover, ecto-5′-nucleotidase expression was increased (80%) at 24 h after hypoxanthine infusion. We suggest that these hypoxanthine-induced biochemical modifications could, at least in part, participate in the pathophysiology of Lesch Nyhan disease.

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1. Introduction

Lesch Nyhan disease is a hereditary X-linked disorder caused by a deficiency of hypoxanthine–guanine phosphoribosyltransferase (HPRT) activity (Nyhan et al., 1965; Henderson, 1968; Rijksen et al., 1981; Jinnah and Friedmann, 2001), in which tissue accumulation of hypoxanthine occurs. Affected patients present cognitive deficits, hyperuricemia, spasticity, dystonia and self-mutilation behavior, characterized by biting of the lips, tongue and fingers with apparent tissue loss (Mizuno, 1986; Jinnah and Gage, 1990; Matthews et al., 1999; Jinnah and Friedmann, 2001). In addition, patients present a prominent loss of striatal dopamine (Jinnah and Friedmann, 2001).

Although the connection between neurological and behavioral dysfunctions present in Lesch Nyhan disease and the

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alteration in the purine metabolism is not elucidated, tissue accumulation of oxypurines such as hypoxanthine has been demonstrated to contribute to the neurological dysfunction present in this disease (Dasheiff, 1980; Kisch et al., 1985; Visser et al., 2000; Ma et al., 2001). In this scenario, a recent study demonstrated that intrastriatal administration of hypoxanthine significantly inhibited Na^+, K^+-ATPase, acetylcholinesterase and catalase activities in a time-dependent manner (Bavaresco et al., 2007). Moreover, it has been demonstrated that hypoxanthine/xanthine oxidase acts as a source of oxidative stress in the vascular system (Oliveira et al., 2001) and might contribute to the destruction of blood-brain barrier observed in ischemic brain tissue (Beckman et al., 1987). A previous study demonstrated that *in vitro* hypoxanthine increased thiobarbituric reactive substances (TBARS) and reduced tissue antioxidant capacity (TRAP) in rat striatum, suggesting an induction of oxidative stress (Bavaresco et al., 2005).

It is well known that the biological role of ATP is not only as metabolic fuel, since ATP and also ATP hydrolysis products (ADP, AMP and adenosine) can act as signaling molecules (Komoszynski and Wojtczak, 1996). In this context, studies indicate that ATP and its metabolites, as well as some purines can act as neuromodulators in different brain regions (Schiffmann and Vanderhaeghen, 1993; Komoszynski and Wojtczak, 1996). It has also been shown that adenosine exerts a regulatory mechanism on the monoamines system (Schiffmann and Vanderhaeghen, 1993; Zhu et al., 2007) and seems to be involved in motor and behavioral changes through its action on specific receptors, named A_1, A_2, A_{2B} and A_3 (Ribeiro et al., 2003; Martinelli and Tuccinardi, 2008). On the other hand, evidence suggests the involvement of the adenosine receptor in aggressive behavior after clonidine administration in mice (Ushijima et al., 1984). Extracellular concentrations of adenosine depend on a regulatory mechanism between the release of this compound from the intracellular medium and the catabolism of ATP, ADP and AMP by ectonucleotidases, such as NTPDases (nucleoside triphosphate diphosphohydrolase) and ecto-5′-nucleotidase (CD73) (Zimmermann, 2006).

NTPDases are membrane bound enzymes with catalytic sites located in the extracellular medium that are responsible for ATP and ADP breakdown. These enzymes are involved in the modulation of synaptic transmission, adult neurogenesis and thromboregulation (Zimmermann, 2006). In the central nervous system (CNS), three different NTPDases have been demonstrated, each differing with regard to substrate preference: NTPDase1 hydrolyzes ATP and ADP about equally well, NTPDase2 stands out for its high preference for ATP and NTPDase3 has a preference for ATP over ADP (Zimmermann, 2006). Moreover, ecto-5′-nucleotidase is a surface-located ecto-enzyme, anchored to the plasmatic membrane via a glycosyl phosphatidylinositol (GPI) anchor and is responsible for extracellular hydrolysis of AMP to adenosine (Zimmermann et al., 1998).

It has been suggested that a deficit in adenosine-mediated neuronal modulation could be involved in the pathological basis of Lesch Nyhan disease (Prior et al., 2006). In this context, Torres et al. (2004) demonstrated that hypoxanthine altered adenosine transport in peripheral blood lymphocytes from both control and Lesch Nyhan patients. In addition, Pesi et al. (2000) showed that cytosolic 5′-nucleotidase activity was increased in erythrocytes from individuals with Lesch Nyhan disease, suggesting the participation of oxypurine toxicity in Lesch Nyhan disease. Moreover, urinary excretion of adenosine was shown to be decreased in patients with Lesch Nyhan disease (Sweetman and Nyhan, 1970).

In the present study, we investigated the effect of intrastriatal hypoxanthine injection on ectonucleotidase activities. We also determined the relative expression of ectonucleotidases in rat striatum. The effect of pre-treatment with vitamins E and C on the hypoxanthine-mediated effects on ectonucleotidases and thiobarbituric acid reactive species (TBARS) were also investigated. Hypoxanthine was injected into the striatum because evidence suggests that many neurobehavioral features of the Lesch Nyhan disease results from the dysfunction of this cerebral structure (Visser et al., 2002).

2. Results

2.1. Experiment 1: effect of intrastriatal hypoxanthine infusion on ATP, ADP and AMP hydrolysis in the synaptosomes of the striatum of rats

Fig. 1 shows the effect of intrastriatal injection of hypoxanthine on nucleotide hydrolysis in the striatum of rats at different periods after oxypurine administration. As can be seen, ATP [$t(6)=3.200$; $p<0.01$] (Fig. 1A), ADP [$t(6)=2.828$; $p<0.01$] (Fig. 1B) and AMP [$t(6)=3.551$; $p<0.01$] (Fig. 1C) hydrolysis were significantly inhibited 24 h after hypoxanthine infusion, but not at 30 min {ATP [$t(6)=0.739$; $p>0.05$] (Fig. 1A); ADP [$t(6)= 1.395$; $p>0.05$] (Fig. 1B); AMP [$t(6)=0.563$; $p>0.05$] (Fig. 1C)} or 7 days {ATP [$t(6)=1.042$; $p>0.05$] (Fig. 1A); ADP [$t(6)=0.695$; $p>0.05$] (Fig. 1B); AMP [$t(6)=0.543$; $p>0.05$] (Fig. 1C)}. Nucleotide hydrolysis was not altered after hypoxanthine administration in contralateral striatum (data not shown).

2.2. Experiment 2: relative expression of striatal NTPDases and ecto-5′-nucleotidase, analyzed by semi-quantitative RT-PCR

Since nucleotide hydrolysis was only inhibited at 24 h after hypoxanthine administration, we also analyzed the relative expressions of NTPDases (NTPDase1, NTPDase2 and NTPDase3) and ecto-5′-nucleotidase in rat striatum after 24 h of hypoxanthine administration. As can be seen in Fig. 2, the relative expressions of NTPDase 1 (Figs. 2A–B), NTPDase 2 (Figs. 2C–D), and NTPDase 3 (Figs. 2E–F) were not altered by hypoxanthine treatment. However, the relative expression of ecto-5′-nucleotidase (Figs. 2G–H) was significantly increased (80%) after oxypurine administration.

2.3. Experiment 3: effect of pre-treatment with vitamins E and C on the ectonucleotidase activities and on TBARS in rat striatum

In this set of experiments, the effect of pre-treatment with vitamins E and C was determined on the effects elicited by 24 h

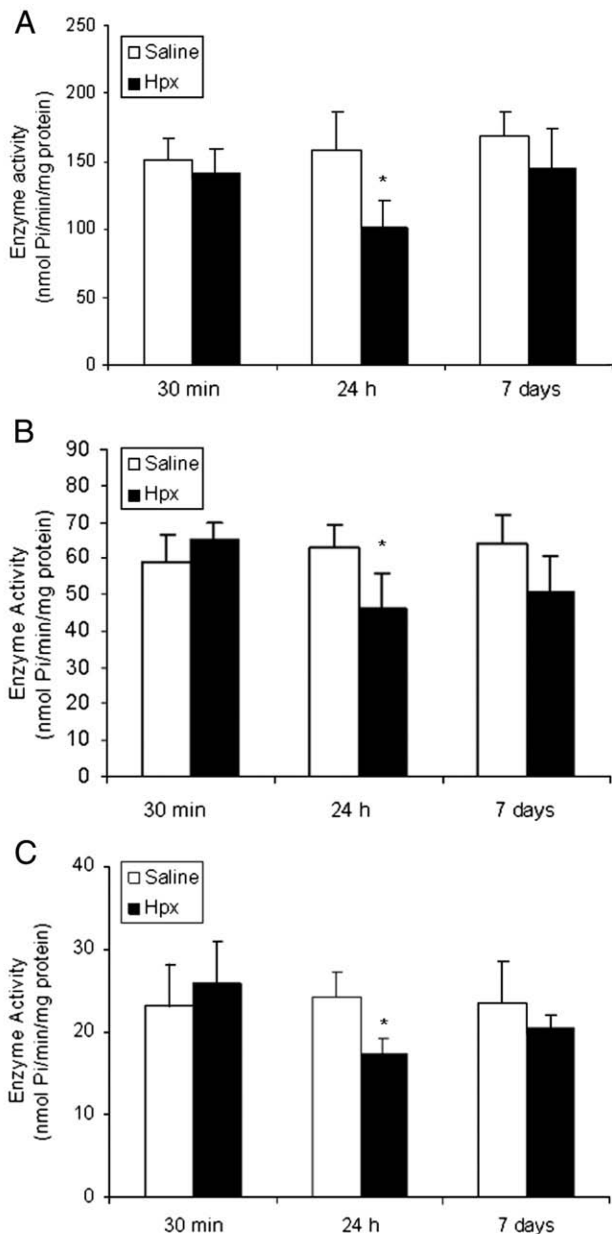

Fig. 1 – Effect of intrastriatal hypoxanthine injection on ATP (A), ADP (B) and AMP (C) hydrolysis in the striatum of rats at different times after infusion. Data are means ± SD for four animals in each group. *$p < 0.05$ compared to sham group (Student's *t*-test). Hpx — hypoxanthine.

intrastriatal injection of hypoxanthine on nucleotide hydrolysis in striatum of rats. Results revealed that pre-treatment with vitamins E and C prevents the inhibitory effect on ATP, ADP and AMP hydrolysis mediated by hypoxanthine infusion [$F(3,12) = 4.425; p < 0.05$] (Fig. 3).

In addition the effect of vitamins E and C administration on the lipid peroxidation caused by hypoxanthine infusion in the TBARS assay was also observed. As can be seen in Fig. 4, pre-treatment with these antioxidants was able to prevent the increased TBARS elicited by hypoxanthine administration [$F(3,16) = 3.395$; $p < 0.05$].

2.4. *Experiment 4: effect of pre-incubation with hypoxanthine on ATP, ADP and AMP hydrolysis in the synaptosomes of rat striatum*

In order to confirm the indirect effect of hypoxanthine on nucleotide hydrolysis, we tested the effect of pre-incubation with hypoxanthine (10 μM) on ATP (Fig. 5A), ADP (Fig. 5B) and AMP (Fig. 5C) hydrolysis in striatal synaptosomes of rats. Results demonstrated that the pre-incubation with hypoxanthine significantly inhibited ATP [$t(4) = 2.802$; $p < 0.05$], ADP [$t(4) = 3.444$; $p < 0.05$] and AMP [$t(4) = 5.305$; $p < 0.05$] hydrolysis in the striatum of rats.

3. Discussion

Patients affected by Lesch Nyhan disease present several signs and symptoms, including mental retardation, self-mutilation behavior and dysfunction of the dopamine transmitter system of the basal ganglia (Jinnah and Gage, 1990; Jinnah and Friedmann, 2001). Accumulation of hypoxanthine is a common feature of patients with Lesch Nyhan (Rosenbloom et al., 1967; Harkness et al., 1988; Puig and Mateos, 1993). However, the pathophysiology of this disease is still obscure.

NTPDases form a group of enzymes that can hydrolyze ATP and ADP to AMP with different abilities, while 5′-nucleotidase promotes the hydrolysis of AMP to adenosine. Since ATP could act as an excitatory neurotransmitter, dysregulation of the delicate control of ATP hydrolysis could be involved in some neuronal diseases (Berti et al., 2001; Delwing et al., 2005). As such, NTPDase and ecto-5′-nucleotidase activities could be inhibited via a stoichiometric mechanism promoted by ATP and ADP hydrolysis inhibition (Vuaden et al., 2007).

In the present study, we first evaluated the effect of intrastriatal hypoxanthine administration on ectonucleotidase (NTPDases and ecto-5′-nucleotidase) activities in the striatum of rats at different post-infusion times. Results showed that intrastriatal hypoxanthine infusion inhibits the hydrolysis of ATP (35%), ADP (27%) and AMP (29%) at 24 h after oxypurine administration, but not after 30 min or 7 days. The inhibition of ATP hydrolysis observed in this study possibly led to the nucleotide accumulation in the extracellular medium. In this context, studies showed that large amounts of ATP in the extracellular medium could mediate cell death through activation of P2X receptors [44] and may be involved in the induction of the death of different cell types including hepatocytes, microglial and myeloid cells (Tinton et al., 1993; Ferrari et al., 1997). It should be noted that nucleotide hydrolysis returned to basal values at 7 days after intrastriatal hypoxanthine infusion. Since ectonucleotidases can also respond by compensatory alterations in their activities, due to alterations in gene transcription (Vuaden et al., 2007), this modification observed in our study 7 days after hypoxanthine infusion could be a consequence of enzymatic adaptation. However, it has been shown that hypoxanthine diffusion in brain occurs via a high-capacity saturable transport system, which is bidirectional and facilitates the diffusion of this oxypurine (Spector, 1987). Evidence has shown that most of the hypoxanthine infused into the rat brain was degraded before 24 h (Spector, 1988). Therefore, it is

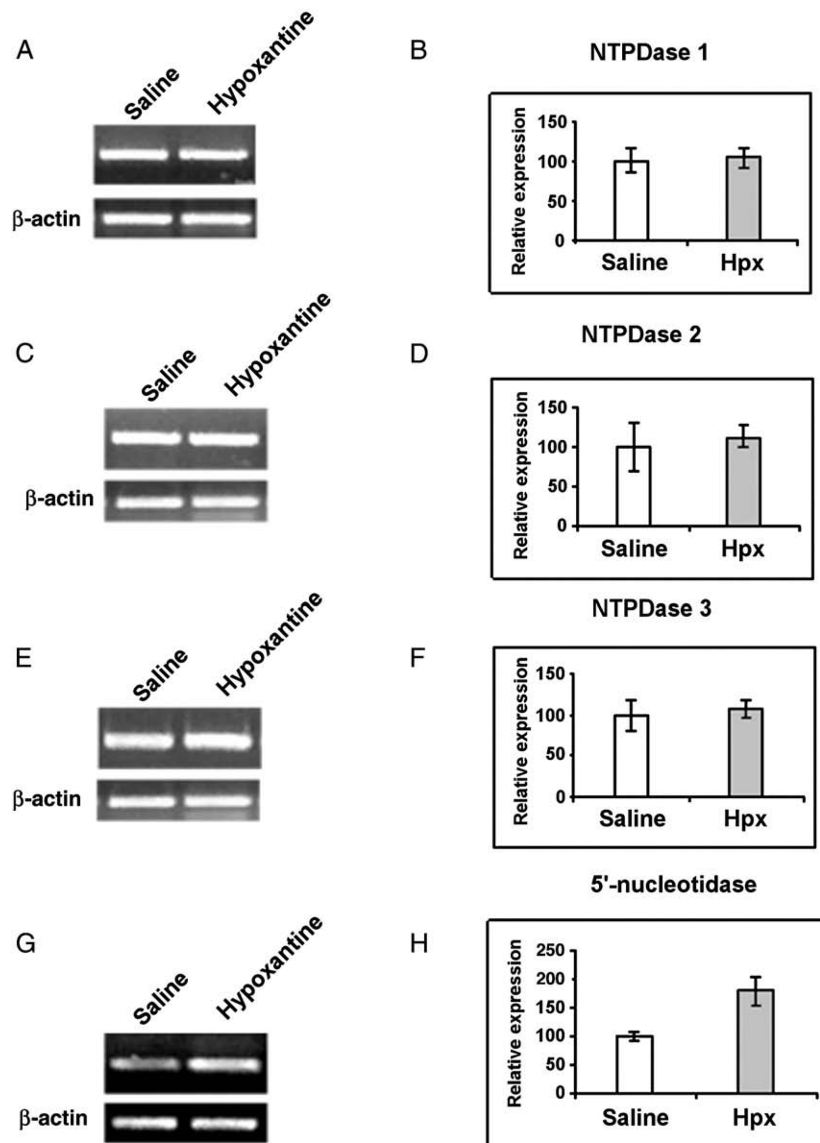

Fig. 2 – Gene expression patterns at 24 h after intrastriatal hypoxanthine injection; NTPDase 1 (A and B), NTPDase 2 (C and D), NTPDase 3 (E and F) and 5′-nucleotidase (G and H) and β-actin in striatum of rats. Data in A, C, E, and G are representative of three individual experiments. The results in the graphs B, D, F, and G were expressed as optical densitometry (O.D.) of the NTPDase-related genes versus β-actin expression (mean ± S.D) of three independent replicate RT-PCR experiments.

possible that the normal hydrolysis observed at 7 days after treatment occurs due to hypoxanthine catabolism and, consequently, due to a decrease of hypoxanthine levels in the brain.

Since the alterations induced by intrastriatal hypoxanthine in NTPDases and ecto-5′-nucleotidase activities could exert modifications in transcriptional control, we decided to investigate the relative expression of NTPDases (NTPDase1, NTPDase2 and NTPDase3) and ecto-5′-nucleotidase in rat striatum at 24 h after hypoxanthine administration. Our results showed that intrastriatal hypoxanthine infusion was able to up-regulate ecto-5′-nucleotidase mRNAs. The exact mechanism for this effect is not known, although the action of a positive feedback autoregulatory loop may be involved. In contrast, Lorenz et al. (2007) showed an increase in NTPDase 3–6 in HPRT-deficient Neuro2a cells. The discrepancy observed in these studies may be due to the different experimental procedure used since, as suggested by Jinnah et al. (1993) and also Brosh et al. (2000), changes in nucleotide concentrations in HPRT-deficient cells may actually be overcompensated by the increase in the rate of de novo synthesis of purine, leading to an abnormal increase of purine and pyrimidine concentrations (Pinto et al., 2005).

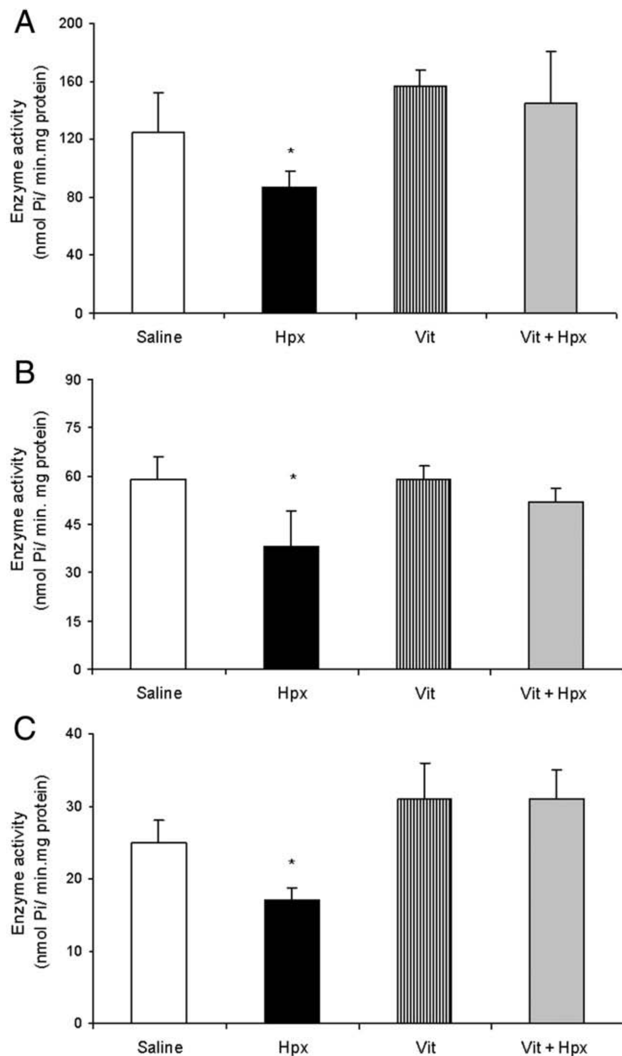

Fig. 3 – Effect of pre-treatment with vitamins E and C on ATP (A), ADP (B) and AMP (C) hydrolysis 24 h after intrastriatal hypoxanthine injection in the striatum of rats. Data are means ± SD of four animals in each group. *$p < 0.05$ compared to sham group (ANOVA followed by Duncan multiple test). Hpx — hypoxanthine.

Moreover, Braun et al. (1997) showed that permanent focal ischemia causes a reactive increase in 5′-nucleotidase on astrocytes and microglial cells. On the other hand, Zimmermann et al. (1998) reported that this up-regulation in ecto-5′-nucleotidase mRNAs could increase the capacity of the tissue to hydrolyze extracellular AMP, possibly derived from ATP liberated by damaged tissue and, consequently, in this case, adenosine could exert a neuroprotective effect. This interpretation is in agreement with those of other authors in the literature (Vuaden et al., 2007; Krishna et al., 2006).

Neychev and Mitev (2004) proposed that modifications in the adenosine system could implicate in CNS alterations, such as self-injuries, similar to those observed in Lesch Nyhan disease. In this perspective, Bertelli et al. (2006) suggested that adenosine neurotransmission might be involved in the specific neurobehavioral features of LND by increased expression of adenosine A1 receptors. Moreover, Torres et al. (2004) suggested that the excess of hypoxanthine in striatum could alter extracellular adenosine concentration, leading to self-injurious biting. However, to date, this hypothesis has not been confirmed.

We also evaluated some possible mechanisms involved in the hypoxanthine-induced inhibition of ectonucleotidases. In this context, our results demonstrated that intrastriatal infusion of hypoxanthine increased TBARS, an index of lipid peroxidation, in rat striatum. Pre-treatment with vitamins E and C prevented the effects caused by hypoxanthine on nucleotide hydrolysis and on TBARS in rat striatum. In contrast, Visser et al. (2002) did not observe alteration in the TBARS in the brain of HPRT-deficient animals (Visser et al., 2002). This discrepancy may be due to the different experimental procedure used. Levels normal of lipid peroxidation observed by these investigators could indicate a compensatory mechanism in order to reduce cell damage. In our study we used an overload of hypoxanthine injected directly into the striatum, where cells would not be adapted to this oxypurine overload.

These results are in agreement with those of other studies and suggest the participation of oxidative stress in the inhibition of NTPDase and ecto-5′-nucleotidase activities in renal epithelial cells and in the brain (Delwing et al., 2005; Siegfried et al., 1996). It has been demonstrated that the brain is highly susceptible to oxidative stress because it has low cerebral antioxidant defenses compared to other tissues (Halliwell, 1996; Floyd, 1999), a fact that makes it more vulnerable to increases in reactive oxygen species.

We have shown that hypoxanthine induces free radical generation (enhanced chemiluminescence) and reduces the antioxidant defenses (decreased TRAP), i.e., elicits oxidative stress in the brain. In the present study, we demonstrated that intrastriatal hypoxanthine infusion increased TBARS in rat striatum and this effect could be prevented by vitamins E and C administration group (Bavaresco et al., 2004; Bavaresco et al., 2005; Bavaresco et al., 2007). Vitamin E is a lipid-soluble vitamin that interacts with cell membranes, reportedly trapping free radicals and interrupting oxidative damage, preventing lipid peroxidation. This vitamin also requires vitamin C (ascorbate) for its regeneration (Zugno et al., 2007). In this scenario, Vietta et al. (1996) demonstrated that trolox, water

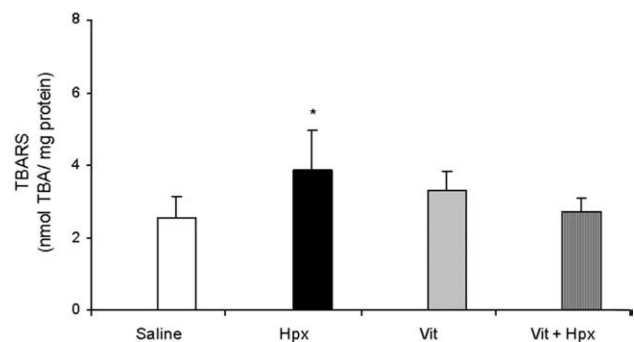

Fig. 4 – Effect of pre-treatment with vitamins E and C on TBARS at 24 h after intrastriatal hypoxanthine injection in the striatum of rats. Data are means ± SD of five animals in each group. *$p < 0.05$ compared to sham group (ANOVA followed by Duncan multiple test). Hpx — hypoxanthine.

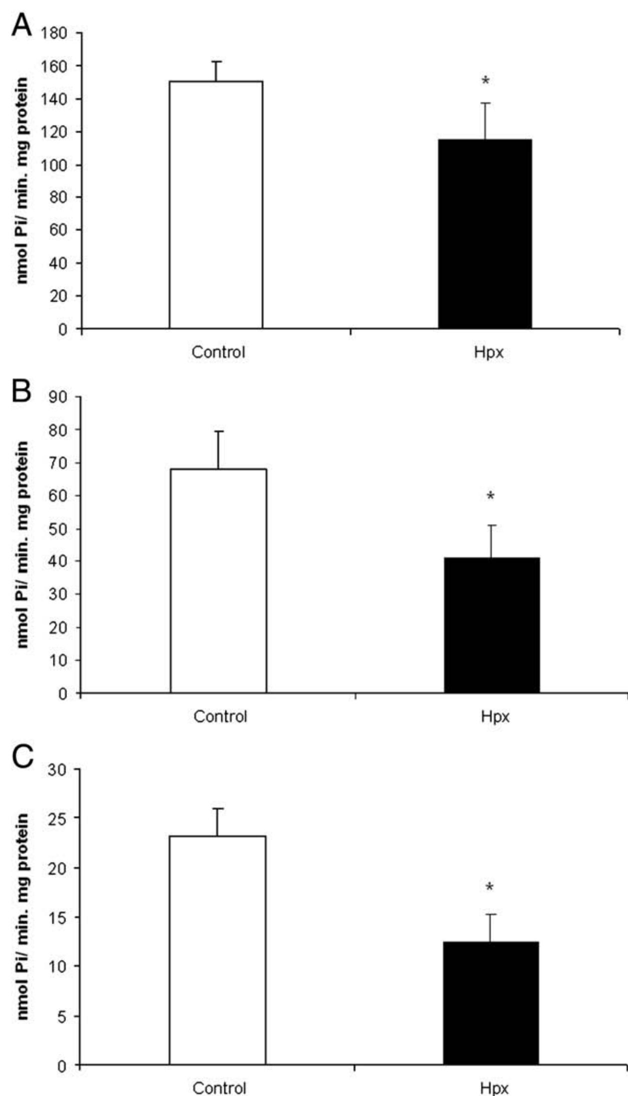

Fig. 5 – Effect of hypoxanthine on ATP (A), ADP (B) and AMP (C) hydrolysis in synaptosomes from the striatum of rats. Striatum homogenates were incubated at 37 °C for 1 h with 10 μM hypoxanthine. ATP, ADP and AMP hydrolysis were determined after synaptosome preparation. Data are means ± SD of four animals in each group. *$p < 0.05$ Hpx — hypoxanthine.

soluble vitamin E, was able to totally prevent lipid peroxidation and ADPase activity inhibition caused by the oxidant generating system in the synaptic plasma membrane.

Our data indicate that hypoxanthine inhibits ectonucleotidase activity by increasing lipid peroxidation and/or oxidative stress, since vitamins E and C prevented such an effect. Furthermore, pre-incubation with hypoxanthine for 1 h before synaptosome preparation was able to inhibit ATP, ADP and AMP hydrolysis, indicating an indirect effect of hypoxanthine on nucleotide hydrolysis, possibly by free radical generation. These results are in agreement with other studies from the literature, which have suggested the participation of oxidative stress in the inhibition of NTPDase and ecto-5′-nucleotidase activities in renal epithelial cells and in the brain (Vuaden et al., 2007; Matute et al., 2007). In these studies the authors showed that arginine and an oxidant generating system ($H_2O_2/Fe^{(2+)}$/ascorbate) inhibited NTPDase activity, probably due to the increase in lipid peroxidation and/or oxidative stress. In our study, pre-treatment with vitamins E and C could prevent the propagation of lipid peroxidation, also acting as a stabilizer of the membranes in which ectonucleotidases are inserted.

Although it is difficult to extrapolate our results to the human condition, it is conceivable that the inhibition of ectonucleotidase activities might be involved in the pathophysiology of the neurological features present in patients with Lesch Nyhan disease. However, more studies are necessary to investigate other mechanisms involved in this metabolic condition.

4. Experimental procedures

4.1. *Animals and reagents*

Sixty-day-old male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22 °C) colony room, with free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of the Brazilian Society for Experimental Biology and was approved by the ethics committee of the Federal University of Rio Grande do Sul, Brazil and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985). All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

4.2. *In vivo studies*

4.2.1. *Experimental treatment*

For stereotaxic surgery and cannula placement, rats were anesthetized with ketamine and xylazine (75 and 10 mg/kg ip, respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge stainless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP — 0.5 mm; ML — 2.5 mm; V — 2.5 mm from the dura) (Paxinos and Watson, 1986). Two days after the surgery, a 30-gauge needle was inserted into the guide cannula in order to inject buffered hypoxanthine (10 μM in saline vehicle) or vehicle (saline) into the right striatum over a 1 min interval. The volume administered (saline or hypoxanthine) was 2 μL. Animals were divided into two groups: group 1 (vehicle group), rats that received intrastriatal saline and group 2 (hypoxanthine-treated), rats that received intrastriatal hypoxanthine solution (20 pmol/2 μL). Only one injection of hypoxanthine was given and the time spent in giving this injection was 1 min. The hypoxanthine concentration was chosen according to Puig et al. (1989). For nucleotidase activities assays, rats were sacrificed at 30 min, 24 h or 7 days after drug infusion. For the analysis of gene expression by semi-quantitative RT-PCR, TBARS and for the mea-

surement of nucleotidase activity assays after the pre-treatment with vitamins E and C, animals were sacrificed 24 h after hypoxanthine or vehicle (saline) injections. Four rats were used in each experimental group.

4.3. *Tissue preparation*

Animals were killed by decapitation without anesthesia, the brain was removed and striatum were dissected out. For the nucleotidase assay, brains were placed in ice-cold isolation medium (320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA), and striatum was immediately dissected on ice. The striatum was gently homogenized in 5 volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer and synaptosomes were isolated, as previously described (Nagy and Delgado-Escueta, 1984).

4.4. *Synaptosome preparation*

Synaptosomes were isolated as previously described (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 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 washed twice at 15,000 *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll, and the synaptosome pellet was then 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. The synaptosomal fraction was used for assays immediately after the preparation. The integrity of the synaptosome was confirmed by determining lactate dehydrogenase (LDH) activity, using the Labtest kit (Labtest, Lagoa Santa, MG, Brazil).

In a second set of experiments, the homogenate of the striatum was separately pre-incubated at 37 °C for 1 h with 10.0 μM hypoxanthine, which was dissolved in 5.0 mM HEPES buffer, pH 7.45, followed by synaptosome preparation.

4.5. *Determination of NTPDase and 5′-nucleotidase activities in synaptosomes of striatum*

The reaction medium used to assay ATP and ADP hydrolysis was essentially as previously described (Delwing et al., 2007) 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 Mg Cl_2, 100 mM Tris–HCl, pH 7.5, and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984).

The synaptosome preparation (10–20 μg protein) was added to the reaction medium and pre-incubated 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 and stopped by the addition of 200 μl trichloroacetic acid (10%). The samples were chilled on ice for 10 min, and 100 μl samples were taken for the assay of inorganic phosphate (Pi) released using a colorimetric method (Chan et al., 1986). Incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid (final concentration 5%) were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

4.6. *Analysis of gene expression by semi-quantitative RT-PCR*

The analysis of the expression of NTPDase1, NTPDase2, NTPDase3 and ecto-5′-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twenty-four hours after intrastriatal injection of hypoxanthine or vehicle (saline), the striatum of rats was isolated for total RNA extraction with Trizol reagent (Invitrogen), in accordance with the manufacturer's instructions. The cDNA species were synthesized with Super Script First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA and oligo (dT) primer, according to the suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (0.1 μl) was used as a template for PCR with specific primers for NTPDase1, NTPDase2, NTPDase3 and ecto-5′-nucleotidase. β-actin PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 μl) using a concentration of 0.4 μM of each polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCRs were as follows: Initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step (NTPDase1, NTPDase3 and ecto-5′-nucleotidase: 65 °C; NTPDase2: 66 °C; β-actin: 58.5 °C), 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. The amplification products were: NTPDase1 — 543 bp; NTPDase2 — 331 bp; NTPDase3 — 267 bp; ecto-5′-nucleotidase — 405 bp; β-actin — 210 bp. For each set of PCR reactions, negative controls were included. Five microliters of the PCR reaction was analyzed on a 1% agarose gel, containing ethidium bromide and visualized with ultraviolet light. Band intensities were analyzed by Kodak 1D v.3.5.4 software. The following sets of primers were used: for NTPDase1: 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ and 5′AAG ACA CCG TTG AAG GCA CACA TGG-3′; for NTPDase2: 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ and 5′ATT GAA GGC CCG GGG ACG CTG AC-3′; for NTPDase3: 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ and 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; for ecto-5′-nucleotidase: 5′-CCC GGG GGC CAC TAG CAC CTC A-3′ and 5′-GCC TGG ACC ACG GGA ACC TT-3′; for β-actin: 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′.

Each experiment was repeated three times using RNA isolated from independent extractions. The expression analysis was performed in replicate and representative findings were shown.

4.7. *Pre-treatment with vitamins E and C*

In this set of experiments, the animals were pre-treated daily during 7 days with intraperitoneal administration of alpha-tocopherol (40 mg/kg) and ascorbic acid (100 mg/kg). Control

animals received saline. Doses of vitamins E and C were chosen according to Wyse et al. (2002).

4.8. TBARS (thiobarbituric acid reactive species)

To determinate lipid peroxidation, we measured the formation of the thiobarbituric acid reactive species (TBARS), using a method described by Esterbauer and Chesseman (1990). TBARS were determined by the absorbance at 535 nm.

4.9. Protein determination

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

4.10. Statistical analysis

Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA) followed by the Duncan multiple range tests when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC compatible computer. Values of $P < 0.05$ were considered to be significant.

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