Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats

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A R T I C L E   I N F O
Article history:
Received 24 March 2012
Accepted 19 September 2012

Keywords:
Anthocyanins
Scopolamine
Ectonucleotidases
Memory

A B S T R A C T
Aims: We investigated whether the treatment with anthocyanins prevents the scopolamine-induced memory deficits and whether ectonucleotidase activities and purine levels are altered in the cerebral cortex (CC) and hippocampus (HC) in this model of mnemonic deficit in rats.

Main methods: The animals were divided into 4 experimental groups: control (vehicle), anthocyanins (Antho), scopolamine (SCO), and scopolamine plus anthocyanins (SCO+Antho). After seven days of treatment, they were tested in the inhibitory avoidance task and open field test and submitted to euthanasia. The CC and the HC were collected for biochemical assays. The effect of treatment with Antho (200 mg kg⁻¹, i.p.) was investigated in rats trained to a stable level of performance and post-treated with SCO (1 mg kg⁻¹, i.p. 30 min after training).

Key findings: The treatment with SCO decreased the step-down latency in inhibitory avoidance task. Antho prevented the scopolamine-induced memory impairment and also the increase of NTPDase activity in the CC and HC. Furthermore, the treatment with anthocyanins prevents the decrease in 5′-nucleotidase activity and the increase in adenosine deaminase activity induced by SCO in HC. In addition, the treatment with Antho prevented the decrease in ATP levels induced by SCO in the CC and HC.

Significance: Our results show that scopolamine may affect purinergic enzymatic cascade or cause alterations in energy metabolism inducing loss of memory. In contrast Antho could reverse these changes, suggesting a neuroprotective effect of Antho on ectonucleotidase activities and neuronal energetic metabolism.

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Introduction

The extracellular nucleotide ATP and its nucleoside derivative adenosine are important signaling molecules involved in innumerable physiological and pathological functions (Bours et al., 2006; Burnstock, 2006a). It has been shown that ATP and adenosine have an array of functions in the central nervous system (CNS) acting as neurotransmitters and activating purinergic and adenosinergic receptors, respectively (Burnstock, 2011; Zimmermann, 1996, 1999, 2006). The levels of extracellular ATP and adenosine may be related to processes of learning and memory formation, since various evidences point to LTP and LTD and synaptic plasticity as a neural basis for cognitive processes (Cooke and Bliss, 2006; Howland and Wang, 2008; Løvinger, 2010; Malenka, 1994).

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The extracellular levels of ATP and adenosine are regulated by a cascade of cell-surface-bound enzymes named ectonucleotidases (Battastini et al., 1991). The NTPDase is an enzyme that hydrolyzes ATP and ADP into AMP, which is subsequently converted to adenosine by the enzyme 5′-nucleotidase (Battastini et al., 1991; Zimmermann, 1996, 1999, 2006). Moreover, adenosine is cleared by the enzyme adenosine deaminase in inosine in the synaptic cleft (Robson et al., 2006, 2005). Together, these enzymes constitute an organized enzymatic cascade for the regulation of nucleotide-mediated signaling, controlling rate, degradation, and nucleoside formation (Abbracchio et al., 2009; Burnstock, 2009, 2011; Schetinger et al., 2007, 2001). The involvement of ectonucleotidases on the process of learning and memory in rats has also been described (Bonan et al., 1998, 2000; Pereira et al., 2002).

Scopolamine (SCO) is a non-selective muscarinic cholinergic antagonist which produces a transient memory impairment in rodents (Klinkenberg and Blokland, 2010). It has been also reported that SCO reduces frontal cortex perfusion in young humans (Honer et al., 1988) and impairs the energetic metabolism reducing the ATP levels in the cerebral cortex of rats (Blin et al., 1994; Ray et al., 1992). The impairment of mitochondrial function and reduction of ATP levels...
Anthocyanins belong to the flavonoid family; they present phenolic groups in their chemical structure and give colors to flowers and fruits of a great variety of plants (Veitch and Grayer, 2008; Williams and Grayer, 2004; Yoshida et al., 2009). It has been shown that anthocyanins are potent antioxidants (Kahkonen and Heinonen, 2003; Kahkonen et al., 2001) and have neuroprotective properties (Del Rio et al., 2010), preventing neurotoxicity induced by reperfusion damage model of cerebral ischemia (Min et al., 2011; Shin et al., 2006), by deleterious effects found in models of Parkinson's (Kim et al., 2010) and Alzheimer's disease (AD) (Shih et al., 2010), and have neuroprotective properties (Del Rio et al., 2010), preventing neurotoxicity induced by reperfusion damage model of cerebral ischemia (Min et al., 2011; Shin et al., 2006), by deleterious effects found in models of Parkinson's (Kim et al., 2010) and Alzheimer's disease (AD) (Shih et al., 2010). Altogether these evidences suggest that flavonoid compounds also have beneficial effects on memory and cognition (Spencer, 2010). In fact, it has been described that anthocyanins improve memory in old rats in Morris water maze (Andres-Lacueva et al., 2005) and also cognition in older humans (Krikorian et al., 2010).

In this context, we sought to investigate if anthocyanin treatment prevented the scopolamine-induced memory deficits and if the ectonucleotidase enzymes as well as the ATP and adenosine levels are involved.

Materials and methods

Chemicals

Nucleotides, Trizma Base, Percoll, and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co (St. Luis, MO, USA). Scopolamine HCl (SCO) was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), and anthocyanins were extracted and purified from grape skin and gently donated by Christian Hansen A/S. All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

This study used three months old adult male Wistar rats (350–400 g). They were kept in the Central Animal House of Federal University of Santa Maria in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity of 45–55% with 12 h light/dark cycles. They had free access to standard rodent pelleted diet and water ad libitum. Experiments were conducted in accordance with the Institutional Ethical Committee of the Federal University of Santa Maria.

Exposure

Seven to ten animals per group were usually tested in the experiments (Scheme 1). Rats were treated i.p. with Antho (200 mg kg⁻¹ body weight) daily for 7 days (around 10 a.m.). The doses were calculated as free base form. Scopolamine was dissolved in saline and injected i.p. at a dose of 1 mg kg⁻¹, and administered 30 min after the training in inhibitory avoidance apparatus in accordance with that previously described (Ali and Arafa, 2011). The dose of anthocyanins was chosen on the basis of previous studies indicating neuroprotection (Manach et al., 2004; Saija et al., 1990; Varadinova et al., 2009). In addition, the daily intake of anthocyanins in the residents of the United States is estimated to be about 200 mg or about 9-fold higher than that of other dietary flavonoids, and this also served as a basis for this study (Manach et al., 2004; Wang and Stoner, 2008).

Behavioral procedure

Inhibitory avoidance task

In the final day of treatment with anthocyanins (7th day), the animals were subjected to training in a step-down inhibitory avoidance apparatus in accordance with that previously described (Rubin et al., 2000), and then the animals received SCO (i.p. 1 mg kg⁻¹) 30 min after training. Next, 24 h after the training the animals were subjected to test in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 × 25 × 35-cm box with a grid floor whose left portion was covered by a 7 × 25-cm platform, 2.5 cm high. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3-s 0.4-mA shock was applied to the grid. Retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 300 s was established.

Open field

Immediately after the inhibitory avoidance test session, the animals were transferred to an open-field measuring 56 × 40 × 30 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open field session lasted for 5 min and during this time, an observer, who was not aware of the pharmacological treatments, recorded the number of crossing responses and rearing responses manually. This test was carried out to identify motor disabilities, which might influence inhibitory avoidance performance at testing.

Foot shock sensitivity test

Reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed and was used to determine the flinch and jump thresholds in experimentally naïve animals (Berlese et al., 2005; Rubin et al., 2000). The animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1 s) delivered at 10 s intervals. Shock intensities ranged from 0.1 to 0.5 mA in 0.1 mA increments. The adjustments in shock intensity were made in accordance with each animal's response. The intensity was raised by one unit when no response occurred and lowered by one unit when a response was made. A flinch
response was defined as withdrawal of one paw from the grid floor, and a jump response was defined as withdrawal of three or four paws. Two measurements of each threshold (flinch, and jump) were made, and the mean of each score was calculated for each animal.

Brain tissue preparation

After behavioral tests, the animals were anesthetized under halothane atmosphere before being killed by decapitation and brain were removed and separated into cerebral cortex (CC) and hippocampus (HC) and placed in a solution of Medium I (320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5) on ice (Gutierres et al., 2012). The brain structures were gently homogenized in a glass potter in Medium I. Protein was determined by the Coomassie Blue method with that previously described (Bradford, 1976), using bovine serum albumin as standard solution.

Synaptosome preparation

Synaptosomes were isolated essentially as that previously described (Nagy and Delgado-Escueta, 1984), using a discontinuous Percoll gradient. The CC and HC were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000 × g for 10 min. An aliquot of 0.5 ml of the crude mitochondrial pellet was mixed with 4.0 ml of an 8.5% Percoll solution and layered into an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10%/Percoll solution and layered into an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10%/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an albumin as standard solution.

Assay of lactate dehydrogenase

The integrity of the synaptosomes preparations was confirmed by determining the lactate dehydrogenase (LDH) activity which was obtained after synaptosome lysis with 0.1% Triton X-100 and comparing it with an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brasil).

Assay of NTPDase and 5′-nucleotidase activities

The NTPDase enzymatic assay of the synaptosomes was carried out in plates with a reaction medium containing 5 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 as described in a previous work from our laboratory (Schetinger et al., 2000). The 5′-nucleotidase activity was determined with that previously described (Heymann et al., 1984) in a reaction medium containing 10 mM MgSO4 and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 μl. In synaptosomes 20 μl of enzyme preparation (8–12 μg of protein) was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP to obtain a final concentration of 1.0 mM and incubation proceeded for 20 min. For AMP hydrolysis, the 5′-nucleotidase activity was carried out as previously described and the final concentration of the nucleotide AMP added was 2 mM. The reactions were stopped by the addition of 200 μl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed using malachite green as the colorimetric reagent and KH2PO4 as standard (Chan et al., 1986). All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein. Adenosine deaminase activity was estimated spectrophotometrically by the method of Giusti (1974), and the values were expressed as U/L of protein for ADA.

Analysis of purine levels in cerebral cortex and hippocampus by high pressure liquid chromatography

Sample preparation

ATP and its breakdown products were extracted with that previously described (Ryder, 1985). Briefly, different amounts of the CC or HC were weighted and homogenized with 0.6 M perchloric acid at 0 °C for 1 min with an Ultra-turrax homogenizer (model T 18, IKA® Works Inc., Wilmington, Del., USA). The homogenate was centrifuged at 2000 × g for 10 min, and the supernatant was immediately neutralized to pH 6.5 to 6.8 with 1 M potassium hydroxide.

High performance liquid chromatography analysis

High performance liquid chromatography (HPLC) was performed with a Shimadzu (Kyoto, Japan) equipment composed of a reciprocating pump model LC-20AT, a degasser model DGU-20A5, a diode array detector (DAD) model SPD-M20A, auto-sampler (SIL-20A) and integrator model CBM-20A, operated by the LC Solution 1.22 SP1 software. Separation was achieved with a Phenomenex Synergi 4 μm Fusion RP-80A column (150 × 4.60 mm, 4 μm) with precolumn, using 0.04 M potassium dihydrogen orthophosphate (KH2PO4) and 0.06 M dipotassium hydrogen orthophosphate (K2HPO4) as mobile phase A and acetonitrile as mobile phase B. A gradient elution was used according to the specifications previously described (Scherer et al., 2005), at a flow rate of 0.7 ml/min. Mobile phases were filtered through a 0.45 μm Millipore filter prior to analysis, and all the reagents utilized were of HPLC grade. Purines in the samples (ATP, ADP, AMP and adenosine) were identified by their retention times and DAD spectrum (in the range 200–400 nm), and quantified by comparison of the peak’s area with standards. The results are expressed by pmol of the different compounds per ml of sample.

Protein determination

Protein was measured by the Coomassie Blue method with that previously described (Bradford, 1976), using bovine serum albumin as standard.

Statistical analysis

Statistical analysis of latency test was carried by Scheirer–Ray–Hare (extension of the Kruskal–Wallis test or two-way ANOVA). Foot shock sensitivity test was analyzed by unpaired t test. Enzymatic activity, nucleotide and nucleoside levels, crossing and rearing responses were analyzed by one- or two-way ANOVA, followed by Tukey’s multiple range tests. P<0.05 was considered to represent a significant difference in all experiments.

Results

Behavioral tests

Anthocyanins prevent the impairment of memory induced by scopolamine

Fig. 1 shows the effect of the treatment with anthocyanins (Antho) and scopolamine-induced (SCO) memory deficits, on step-down latencies. Statistical analysis of testing (nonparametric two-way ANOVA) showed a significant scopolamine (1 mg kg−1) vs anthocyanins (200 mg kg−1) or vehicle interaction (Control), revealing that treatment with anthocyanins reverses the impairment of memory induced by SCO [H = 9.75; P = 0.01]. Statistical analysis of training (one-way ANOVA) showed no difference between groups [F(3,31) = 0.77;
P 0.05]. Because motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether SCO or Antho affected shock threshold, or locomotor ability of the animals. Statistical analysis of open-field data (one-way ANOVA) revealed that scopolamine did not alter the number of crossing [F(3,31) = 0.99, P > 0.05] or rearing [F(3,31) = 0.13, P > 0.05] responses in a subsequent open-field test session, suggesting that neither SCO nor Antho caused gross motor disabilities or altered foot shock sensitivity (Table 1). Moreover, SCO did not alter foot shock avoidance, as demonstrated by the similar P > 0.05 responses in a subsequent open-field test session, suggesting that neither SCO nor Antho caused gross motor disabilities or altered foot shock sensitivity (Table 1).

Enzymatic activities

**Anthocyanins prevent the increase in NTPDase activity induced by scopolamine**

Fig. 2 shows the effect of Antho and SCO on the activity of NTPDase in the CC and HC of rats. SCO increased the NTPDase activity in the CC (Fig. 2A) and HC (Fig. 2B) using ATP nucleotide as substrate. Antho prevented the increase in NTPDase activity induced by SCO in the CC [F(1,20) = 7.371; P 0.05, Fig. 2A] and HC [F(1,20) = 6.397; P 0.05, Fig. 2B]. SCO also increased the NTPDase activity in HC using ADP nucleotide as substrate and Antho prevents this effect [F(1,20) = 6.397; P 0.05, Fig. 2D]. No significant differences in the activity of NTPDase in the CC were observed in the groups [F(1,20) = 0.002; P 0.05, Fig. 2C].

**Anthocyanins prevent the increase in adenosine deaminase activity induced by scopolamine**

Fig. 3 shows the effect of Antho and SCO on the activity of adenosine deaminase in the CC (Fig. 3A) and HC (Fig. 3B) of rats. SCO increased the adenosine deaminase activity in the HC. Antho prevented the increase in adenosine deaminase activity induced by SCO in the HC [F(1,20) = 4.897, P 0.05, Fig. 4B]. No significant differences in the activity of adenosine deaminase in the CC was observed [F(1,20) = 0.071, P 0.05, Fig. 4A].

**Content of nucleotides and nucleosides of adenine**

**Anthocyanins can restore decrease in purine levels induced by scopolamine**

Table 2 shows the effect of Antho and SCO on the nucleotide levels in the CC of rats. SCO decreased the ATP levels CC in relation to the all groups. However, when the animals received anthocyanins (SCO + Antho), the levels of ATP were similar to those of the vehicle group [F(1,12) = 14.23; P 0.01]. No significant differences in ADP [F(1,12) = 0.036; P 0.05], AMP [F(1,12) = 3.002; P 0.05] and adenosine levels were observed [F(1,12) = 4.013; P 0.05].

Table 3 shows the effect of Antho and SCO on the nucleotide levels in the HC of rats. SCO decreased the ATP levels in the HC in relation to all the groups. However, when the animals received anthocyanins (SCO + Antho), the levels of ATP were similar to those of the vehicle group [F(1,12) = 5.648; P 0.05]. No significant differences in ADP [F(1,12) = 3.593; P 0.05], AMP [F(1,12) = 1.684; P 0.05] and adenosine [F(1,12) = 0.373; P 0.05] levels were observed.

Discussion

In the present study, we have evaluated the potential preventive role of the anthocyanins (Antho) in the scopolamine-induced memory deficits in rats. Our results have shown that scopolamine impaired the memory consolidation in rats trained in the inhibitory avoidance task. Interestingly, in this study we found that anthocyanins at a dose of 200 mg kg^{-1} during 7 days did not improve the memory of rats in the inhibitory avoidance task, but it prevented the memory deficits induced by scopolamine (SCO) (Scheme 1).

SCO is an alkaloid derived from *Atropa belladonna* (Zhang et al., 2008) and acts as a competitive antagonist of the muscarinic acetylcholine receptor (mACHR) (Wang et al., 2003). For this reason, SCO is used to compromise cholinergic neurotransmission and, in some

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Training</th>
<th>Crossing</th>
<th>Rearing</th>
<th>Flinch (mA)</th>
<th>Jump (mA)</th>
<th>Vocalization (mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>7.50 ± 1.99</td>
<td>21.75 ± 3.13</td>
<td>16.00 ± 2.28</td>
<td>0.36 ± 0.01</td>
<td>0.45 ± 0.02</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>Antho</td>
<td>8.37 ± 1.79</td>
<td>17.25 ± 2.19</td>
<td>13.63 ± 2.09</td>
<td>0.41 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>SCO</td>
<td>5.30 ± 1.59</td>
<td>22.10 ± 2.57</td>
<td>18.00 ± 2.96</td>
<td>0.34 ± 0.01</td>
<td>0.43 ± 0.02</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>SCO+Antho</td>
<td>8.22 ± 1.35</td>
<td>23.89 ± 3.04</td>
<td>20.22 ± 3.30</td>
<td>0.37 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.41 ± 0.03</td>
</tr>
</tbody>
</table>

Statistical analysis: [F(3,31) = 0.77; P > 0.05] [F(3,31) = 0.99; P > 0.05] [F(3,31) = 1.30; P > 0.05] [F(3,31) = 4.48; P > 0.05] [F(3,31) = 1.11; P > 0.05]

Data are means ± SEM for 6–10 animals in each group.
ways, mimics the memory deficit observed in diseases characterized by impairment in cholinergic neurotransmission, such as AD (Christensen et al., 1992; Kopelman and Corn, 1988; Wesnes et al., 1991).

It has been reported that dietary supplementation of antioxidant-rich berries (e.g., blueberry, strawberry) can improve the learning and memory in the aged animal (Williams and Grayer, 2004; Williams et al., 2008). The daily intake of anthocyanin chosen for this study (200 mg kg$^{-1}$) is in agreement with the consumption of that in the residents of the United States population (Manach et al., 2004; Wang and Stoner, 2008). Moreover, previous studies indicate that a daily intake of 200 mg/kg of anthocyanins has protective effects in rats, mice and humans (Bao et al., 2008a, 2008b; Choi et al., 2007; Heinonen, 2001) and improve learning and memory in female rats ovariectomized (Varadinova et al., 2009). Furthermore, there are few studies using acute treatment with anthocyanins, because diverse number of flavonoid compounds, including anthocyanins, seem to require a short-term or long-term to accumulate and promote beneficial effects in the brain of rodents (Barros et al., 2006; Hassimotto and Lajolo, 2011; Ke et al., 2011; Min et al., 2011; Yang et al., 2011). Acute doses of anthocyanins may result in immediate beneficial changes on the peripheral homeostasis and biochemical tissue parameters (Matsumoto et al., 2001; Rossi et al., 2003). However, no previous study of acute and subacute doses of anthocyanins associated with learning and memory have been observed at this point.

Additionally, studies have shown that anthocyanins have the ability to improve memory of old rats in Morris water maze (Andres-Lacueva et al., 2005), and of mice in the inhibitory avoidance task (Barros et al., 2006). Interestingly, it was also shown to be a cognitive improvement in older humans (Krikorian et al., 2010). In line with this view, studies with the Cyanidin-3-O-glucopyranoside (Cy-3G), an isolated anthocyanin abundant in colorful vegetables and fruits, has recently been identified as potent neuroprotective phytochemical since this compound protects against $\alpha \beta$-peptide-mediated cytotoxicity in SH-SY5Y neurocytes (Tarozzi et al., 2008, 2007, 2010) and also can reduce cerebral ischemia damage and age-related neuronal deficits (Shin et al., 2006).

Despite SCO's classical use as an amnesic agent, there are a lot of discrepancies in relation to SCO effects in locomotion. Some studies, in fact, challenge the viability of SCO use as a cognitive impairer, questioning if the alterations in behavior are related to peripheral locomotor effects, instead of memory disruption (Klinkenberg and Blokland, 2010). As shown in Table 1, there were no changes in none of the parameters analyzed. We have also shown in a pilot test (data not shown) that

Fig. 2. Effects of Antho and SCO on NTPDase activity in synaptosomes of the cerebral cortex and hippocampus using ATP (A and B) and ADP (C and D) respectively as substrates. Bars represent the mean ± SEM. *Represents a significant difference in all groups, $P<0.05$. (ANOVA two-way, Tukey test).

Fig. 3. Effects of Antho and SCO on 5′-nucleotidase activity in synaptosomes of the cerebral cortex and hippocampus using AMP as substrate. Bars represent the mean ± SEM. *Represents a significant difference in all groups, $P<0.05$. (ANOVA two-way, Tukey test).
exposure to SCO for 1 h immediately before inhibitory avoidance training did not impact training performance, since control and scopolamine-treated animals’ training session latencies did not change.

In the brain, information storage is involved with an increase in efficiency of synaptic stimulatory pathways (Wierszko, 1996; Wierszko and Ehrlich, 1994). Since the first demonstration of ATP-dependent release of CNS stimulation (Holton, 1959), there is a growing interest in the role of ATP and adenosine in synaptic transmission (Cunha and Ribeiro, 2000). ATP and adenosine act as neurotransmitters modulating purinergic and adenosinergic receptors, respectively (Zimmermann, 1996, 1999, 2006). It has been reported that ATP and adenosine modulate low term potentiation (LTP) and low term depression (LTD) in neurons (de Mendonca et al., 2002; Fujii, 2004; Wierszko and Ehrlich, 1994; Yamazaki et al., 2003) and also contribute to neuronal synaptic plasticity (Costenla et al., 1999; de Mendonca and Ribeiro, 1997; Wierszko, 1996). These processes are related with learning and memory formation (Cooke and Bliss, 2006; Howland and Wang, 2008; Lovinger, 2010; Malenka, 1994).

In CNS, the NTPDase is an important enzyme involved in the purinergic neurotransmission. Our results show that an increase in the NTPDase activity in synaptosomes of the CC and HC in rats treated with SCO leads to a reduction of extracellular ATP in the synaptic cleft, which may impair the purinergic signaling since it reduces the availability of extracellular ATP. In addition, these enzymes differ in their ratios and preferably for the substrate hydrolysis. The NTPDase 1 hydrolyzes ATP and ADP in the same way; also, NTPDases 2, 3 and 8 hydrolyze ATP more than ADP, and NTPDase 4 preferentially hydrolyzes UDP (Zimmermann, 2001). Based on these findings, we believe that the effects found in the hippocampus and cerebral cortex can be related to the activity of different enzyme isoforms. Enzymatic hydrolysis of ATP and ADP in the hippocampus suggests the involvement of NTPDase 1, whereas in the cerebral cortex other isoforms with greater affinity to ATP can be involved (Fig. 2). Furthermore, the reduction on the ATP levels can impair processes like the establishment of the neuronal LTP (Fujii, 2004; Min et al., 2011; Wierszko and Ehrlich, 1994; Yamazaki et al., 2003) affecting memory formation (Cooke and Bliss, 2006; Howland and Wang, 2008; Lovinger, 2010; Malenka, 1994). These results corroborate our findings when compared with the memory evaluated in the inhibitory avoidance task, where the amnesic effect induced by SCO was prevented by the treatment with Antho.

The next step was to assess the 5′-nucleotidase and adenosine deaminase activity. As it can be seen in Fig. 3, SCO decreased the activity of 5′-nucleotidase in synaptosomes of the HC (Fig. 3B), and the treatment with Antho was able to prevent this decrease. Furthermore, our results showed that 5′-nucleotidase and ADA activities seem to be more sensitive in the HC than CC, so we believe that ectonucleotidase in the hippocampus is more affected by scopolamine (see Figs. 3 and 4). However, we can not exclude that specific regions of the cerebral cortex which are known to participate in memory formation, as the posterior cingulate and entorhinal cortex (Lima et al., 2009; Pereira et al., 2005, 2001; Souza et al., 2002), are also affected by SCO administration, so ectonucleotidase activities were measured in the whole cerebral cortex and not in specific regions, might have been the reason that no significant differences were observed in this brain structure. The activity of this enzyme is very important because this leads to production of extracellular adenosine (Burnstock, 2006a, b; Robson et al., 2006; Schetinger et al., 2001). It was also observed that SCO increased the activity of adenosine deaminase, and this effect was prevented by the treatment with Antho. The 5′-nucleotidase and adenosine deaminase are key-enzymes in the regulation of extracellular levels of adenosine in the synaptic cleft. A decrease of 5′-nucleotidase activity reduces the adenosine formation and the increase of adenosine deaminase activity increases the hydrolysis of adenosine to inosine. Thus, the effect of SCO on these enzymes leads to an increased removal of extracellular adenosine decreasing its levels, which may lead to impairment of the adenosinergic neurotransmission.

The depletion of extracellular adenosine can disrupt memory formation since adenosine has been reported as an important neuromodulator in the establishment of LTP and LTD, as well as in synaptic plasticity (de Mendonca et al., 2002), (Costenla et al., 1999; de Mendonca and Ribeiro, 1997; Wierszko, 1996). Based on the activity of the ectonucleotidases, it can be seen that the treatment with SCO increased the hydrolysis of

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**Table 2**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.78±0.089</td>
<td>0.56±0.187</td>
<td>0.40±0.017</td>
<td>0.91±0.128</td>
</tr>
<tr>
<td>Antho</td>
<td>0.83±0.130</td>
<td>0.71±0.040</td>
<td>0.54±0.089</td>
<td>1.14±0.258</td>
</tr>
<tr>
<td>SCO</td>
<td>0.58±0.032</td>
<td>0.59±0.089</td>
<td>0.54±0.057</td>
<td>1.17±0.174</td>
</tr>
<tr>
<td>SCO+Antho</td>
<td>0.90±0.023</td>
<td>0.77±0.052</td>
<td>0.45±0.161</td>
<td>1.07±0.104</td>
</tr>
</tbody>
</table>

Data are means±SEM for 4 animals in each group (cerebral cortex).

**Table 3**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.0±0.044</td>
<td>0.39±0.089</td>
<td>0.22±0.090</td>
<td>0.46±0.087</td>
</tr>
<tr>
<td>Antho</td>
<td>0.93±0.030</td>
<td>0.45±0.064</td>
<td>0.28±0.080</td>
<td>0.54±0.110</td>
</tr>
<tr>
<td>SCO</td>
<td>0.44±0.023 a</td>
<td>0.46±0.059</td>
<td>0.30±0.100</td>
<td>0.59±0.094</td>
</tr>
<tr>
<td>SCO+Antho</td>
<td>0.89±0.043</td>
<td>0.41±0.056</td>
<td>0.26±0.088</td>
<td>0.51±0.098</td>
</tr>
</tbody>
</table>

Data are means±SEM for 4 animals in each group (hippocampus).
extracellular ATP and adenosine. The reduction of these neurotransmitters can reduce the signaling of adenosinergic and purinergic neuro-transmission, respectively, and compromise the acquisition of memory formation. However, studies showing the effect of SCO and Antho on the activity of ectonucleotidases are limited. The possible mechanism by which SCO and Antho act on these enzymes needs to be further investigated.

Studies have shown that SCO impairs energy metabolism and reduces the ATP levels in the CC of rats (Blin et al., 1994; Ray et al., 1992). It is known that worsening of mitochondrial function and reduction of ATP levels are pathological conditions found in neurodegenerative diseases such as AD, which is closely linked to the decline of cognitive processes (Ferrer, 2009; Hauptmann et al., 2009). Other studies also show that SCO reduces the frontal cortex perfusion in young humans (Honer et al., 1988). In addition, it was also observed that intramuscular SCO administration impairs the oxygen consumption and the tissue metabolism of the cardiovascular system and CNS of humans (Kirvela et al., 1994). The glucose treatment was able to prevent deficits on the memory induced by SCO (Stone et al., 1991). Based on these findings, the deleterious effects of SCO could be related to energy depletion in neurons. Thus, the second set of experiments investigated the levels of nucleotides ATP, ADP and AMP and the nucleoside adenosine in the CC and HC in the animals studied.


Acknowledgments

This study was supported by the Christian Hansen LTDA, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.