Arch. Biol. Sci., Belgrade, 65 (1), 33-42, 2013 DOI:10.2298/ABS1301033L

BRAIN CORTICAL INJURY INDUCES CHANGES IN PERIPHERAL LYMPHOCYTE ECTONUCLEOTIDASE ACTIVITIES

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Abstract - Injury and other pathological conditions induce a massive release of ATP and ADP that initiate an immune response. Extracellular nucleotides are degraded by ectonucleotidases: enzymes from E-NTPDase and E-NPP families sequentially hydrolyze ATP and ADP to AMP, which is further hydrolyzed by ecto-5'-nucleotidase to adenosine that exerts suppressive effects on immune cells. We investigated the ectonucleotidase activities of peripheral lymphocytes at different post-injury times after an unilateral brain injury in the rat. Significant and dynamic changes in the lymphocytic ectonucleotidase activities were obtained. ATP- and ADP-hydrolysis changes, together with their calculated ratios, indicate the major contribution of E-NTPDase 1 and its comparable upregulation between sham operation and injury. AMP hydrolysis changes were more brain-injury specific, with a longer-lasting lymphocytic response induced by cortical stab injury (CSI). In summary, CSI and sham operation induce the upregulation of the whole enzyme chain for adenine nucleotide hydrolysis in lymphocytes, suggesting an important roles of ectonucleotidases in the course of recovery after brain injury.

Key words: ATP, ADP, adenosine, E-NTPDase, ecto-5'-nucleotidase brain injury

INTRODUCTION

In the extracellular compartment, purine di- and trinucleotides act as paracrine molecules that regulate the immune response (Di Virgilio, 2005; Bours et al., 2006), platelet aggregation and thrombus formation (Marcus et al., 2003), neurotransmission and muscle contraction (Burnstock, 2007), heart rate (Vassort, 2001) and many other processes. Physiological concentrations of extracellular nucleotides are usually in the nanomolar range (Yegutkin et al., 2006), whilst injury and other pathological conditions, such as inflammation, hypoxia and ischemia, induce the massive release of ATP and ADP from damaged and stressed cells into the extracellular space (Bodin and Burnstock, 1998; Lazarowski et al., 2003). In high concentration, ATP and ADP act as tissue-derived distress signals called damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004) that initiate immune responses (La Sala et al., 2003).

Extracellular nucleotides exert their effects through multiple P2X and P2Y receptors (Ralevic and Burnstock, 1998; Abbracchio et al., 2006) which are widely expressed at immune and non-immune cells (Bours et al., 2011). One of the most studied member of the P2 receptor family is the $P2X_7$ receptor which is apparently involved in sensing danger (Solle et al., 2001). Activation of $P2X_7$ enhances

lymphocytic adhesion and extravasation (Salmi and Jalkanen, 2005) and induces the production of proinflammatory cytokines such as IL-1β, TNF-α, IL-12, IL-18, triggering inflammatory response (Bours et al., 2006).

Extracellular nucleotides are efficiently degraded by the action of ectonucleotidases (Zimmermann, 2000), widely expressed at various immune and non-immune cells, but also present in a soluble form in the extracellular compartment (Yegutkin et al., 2003). Ectonucleotidases belong to four enzyme families: ectonucleoside triphosphate-diphosphohydrolases (E-NTPDase), nucleotide pyrophosphatase/phosphodiesterases (E-NPP), alkaline phosphatase (AP) and ecto-5'-nucleotidase (E-5NT) (Lazarowski et al., 2000; Yegutkin, 2008). Four of 8 members of the E-NTPDase family – E-NTPDases 1, 2, 3 and 8 – are genuine ectoenzymes (Robson et al., 2006) that hydrolyze nucleotides in the range of concentrations that activate P2 receptors, with different ratios regarding the hydrolysis of tri- and dinucleosides (Kukulski et al., 2005). Enzymes that belong to the E-NTPDase and E-NPP families sequentially hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by the action of E-5NT (Zimmermann, 2001).

Adenosine generated in ectonucleotidasic cascade acts as a negative feedback signal counteracting ATP-mediated stimulation by inhibiting proinflammatory cytokine release and promoting IL-10 production (Bours et al., 2006). Adenosine exerts its effect through its own P1 receptor family, ubiquitously present on myeloid and lymphoid cells (Hasko and Cronstein, 2004). Studies with knockout models revealed that the A_{2A} adenosine receptor may have the most important role in downregulation of cell-mediated immunity (Ohta and Sitkovsky, 2001; Thiel et al., 2003; Sitkovsky et al., 2004), by suppressing the proliferation and effector functions of lymphocytes.

In the immune system (Bours et al., 2006), as well as in the vascular compartment (Marcus et al., 2003), E-NTPDase1 (EC 3.6.1.5., CD39) and E-5NT (EC 3.1.3.5., CD73) are designated as key regulators of purinergic signalization. It was demonstrated (Dombrowski et al. 1995; 1998) that E-NTPDase1 mediates cell adhesion and antigen recognition, as well as the cytokine secretion and cytolytic activity of T-cells, whilst for E-5NT, Airas et al., (1998; 2000) showed that it contributes to lymphocytic proliferation and activation, and promotes lymphocytic binding to endothelial vascular cells, enabling extravasation. These findings were supported by the results of experiments with animals deficient in genes for both enzymes (Sun et al., 2011; Mills et al., 2008).

Several neuropathologies, including brain injury, are now considered to be true neuroinflammatory conditions (Morganti-Kossman et al., 2007) owing to their sustained microglial and astroglial activation (Di Virgilio et al., 2009), disruption of the blood brain barrier and entry of lymphocytes from the periphery (Soares et al., 1995; Wang and Dore, 2007). Nevertheless, the results from several groups have shown that various insults to the brain, such as epilepsy (Bruno et al., 2003; Busnello et al., 2008; Grosso et al., 2009) or experimental autoimmune encephalomyelitis (Lavrnja et al., 2009), induce changes in ectonucleotidase activities in the serum. On the other hand, studies with knockout animals that lack the genes for E-NTPDase 1 (Sun et al., 2011; Hyman et al., 2009), or E-5NT (Mills et al., 2008; Thompson et al., 2008; Takedachi et al., 2008), confirmed that their coordinated activity is essential for an adequate immune response in the inflammatory, hypoxic and prothrombotic states that arise as a consequence of brain injury (Jeremitsky et al., 2003). Considering the role of ectonucleotidases in the control of lymphocyte effector functions (Yegutkin et al., 2010; Bours et al., 2006), in this study we have examined ectonucleotidase activities in the isolated fraction of peripheral lymphocytes at different post-injury times after unilateral brain injury in rats.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (55 animals, 3 months old, 250-300 g body weight) were used in this study. The animals were maintained three to a cage at a constant

temperature on a 12 h light/dark cycle with free access to food and water.

Surgical procedure

The experimental protocols were approved by the Institutional Animal Care Committee and were in conformity with the recommendation provided by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A). All efforts were made to minimize the number of used animals and their suffering.

Fifty animals were randomly divided into two groups. Each animal was anesthetized with an intraperitoneal injection of Zoletil®50 (Virbac, France) in a dose of 50 mg/kg body weight; they were positioned into a stereotaxic frame and the scalp was shaved. An incision was made along the midline of the scalp and the bregma was exposed. Cortical stab injury (CSI) was performed as previously described (Nedeljković et al. 2006) by a 1 mm-wide dental drill through the skull on the left side (2 mm lateral from the midline, 2 mm posterior to the bregma). The drill was inserted 1.5 mm below the underlying cortical region. The wound was sterile-closed. Animals in another group were anesthetized, the scalps were shaved and after making the incision along the midline leaving the dura intact, the wound was sutured (sham-operated animals). Animals were placed in a heated room and monitored while recovering from anesthesia. In both CSI and sham-operated groups, the animals (5/time group) were allowed to recover for 4 h, 1, 2, 7 and 14 days after the injury or sham operation. Another five age-matched intact animals was used as a physiological control group.

Isolation of peripheral blood lymphocyte fraction

Lymphocytes were isolated from the rat blood collected with heparin directly from heart and separated on a density gradient as described by Böyum (1968). Lymphocytes were washed 3 times in saline and resuspended to a final concentration of 200 μg/ml in saline.

Trypan blue exclusion test of cell viability (TB test)

The TB test (Strober, 2001) was used to assess the viability of lymphocytes, and groups with viability greater than 95% were used for the experiments. Due to clear morphological distinctions, we determined the contamination of the samples with granulocytes and platelets and used the samples with less than 5% contamination in further experiments.

Protein determination

Protein content was determined by the method described by Bradford (1976), using bovine serum albumin as standard.

Measurement of ATP, ADP and AMP hydrolysis in peripheral blood lymphocyte fraction

Freshly isolated lymphocytes were subjected to ATP-, ADP- and AMP-hydrolysis measurement according to Leal et al., (2005). The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer, pH 8.0, at a final volume of 200 μl. Twenty microliters (≈ 4 μg protein) of intact lymphocyte fraction was added to reaction medium and preincubated for 10 min at 37°C. The reaction was started by the addition of ATP, ADP or AMP at a final concentration of 0.5 mM, incubation lasted for 60 min at 37°C and then was stopped by adding 22 μl of 3M perchloric acid (PCA). Enzyme mixtures were chilled on ice for 10 min and then assayed for inorganic phosphate (Pi) by the method described by Chan et al. (1986), using malachite green as a colorimetric substrate and KH_2PO_4 as standard. All samples were assayed in quintuplicate, with controls for every sample in order to correct for non-enzymatic hydrolysis. The controls were prepared by adding substrate after PCA addition. Specific enzyme activities were reported as nmol Pi released/min/mg protein.

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise indicated.

Data analysis

Results are expressed as means ± SEM. The comparison between time groups was made by Student's *t* test for independent samples, followed by Tukey's test for multiple comparisons among means. The significance was determined at levels $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.001$ (***).

RESULTS

We analyzed nucleotide-hydrolyzing activities in the lymphocyte fractions obtained from animals submitted to CSI or sham operation at different post-injury times and compared them to the activities of the intact control group (Fig. 1A-B, Fig.3). The intact group showed the highest hydrolyzing activity towards ATP (9.2 ± 0.9 nmolPi/min/mg), less for ADP (6.7 ± 0.4 nmolPi/min/mg) and the lowest for AMP (5.9 ± 0.2 nmolPi/min/mg).

During the recovery period, the ATP hydrolyzing activities in the CSI animals were significantly different in respect to the intact control group, but indistinguishable in comparison to the corresponding sham group (Fig. 1A). ATP hydrolysis decreased at 4 h post-injury (72.5 ± 4.9%, $p < 0.05$) and during the next several hours returned to the control levels. Afterwards, ATP hydrolysis significantly increased in both the injured and sham-operated groups in comparison to the intact control: at 2-day (136.6 ± 4.3% , $p < 0.05$) in CSI and (138.6 ± 1.2%, $p < 0.05$) in the sham-operated group, on day 7 (133.2 ± 5.2%, $p < 0.05$) in CSI and (124.8 ± 5.2%, $p < 0.05$) in the sham-operated group, at day 14 (151.5 ± 1.9%, $p < 0.001$) in CSI and (154.5 ± 7.9%, $p < 0.05$) in the sham-operated group.

ADP hydrolysis in the CSI and corresponding sham-operated control groups followed a trend similar to that obtained for ATP hydrolysis (Fig. 1B). In both groups at 4 h ADP hydrolysis decreased slightly, but not significantly in respect to the intact control. At days 2 and 7, ADP hydrolysis both in the CSI and sham-operated groups was significantly higher in respect to the intact control group: at day 2 (164.4 ± 6.9%, $p < 0.001$) in CSI and (150.2 ± 6.6%, $p < 0.005$) in the sham-operated group, at day 7 (123.1 ± 1.7%, $p < 0.005$) in CSI and (142.5 ± 0.9%, $p < 0.05$) in the sham-operated group. At day 14, ADP hydrolysis in CSI returned to the intact control level, with the activities of the corresponding sham group still increased in respect to the intact control (138.4± 1.9%, $p < 0.05$).

ATP/ADP hydrolysis ratios at different post-operative times (Fig. 2), both for CSI and corresponding sham groups, are calculated from the data given in Figs 1.A and B. Hydrolysis ratios both in the CSI and sham-operated groups were indistinguishable from the ratio obtained for the intact control group (1.4±0.2) throughout the observed period.

AMP hydrolysis (Fig. 3) at 4 h remained at the physiological level, both in the CSI and sham groups, while later in the CSI group the hydrolysis significantly changed in comparison to the intact control group: at day 1 (170.9 ± 3.2%, $p < 0.001$), day 2 (126.4 ± 2.5%, $p < 0.005$), day 7 (175.0 ± 1.4%, $p < 0.001$) and day 14 (154.8 ± 7.7 %, $p < 0.005$), post-injury. In contrast to ATP and ADP hydrolysis, significant differences in respect to AMP hydrolysis occured between the CSI and sham-control animals: in CSI at day 2 (66.8 ± 2.15%, $p < 0.001$) and day 7 (146.2 ± 3.14, $p < 0.001$) in comparison to the corresponding sham group. For other time-points, the activities of the CSI animals were indistinguishable from the sham-operated group.

DISCUSSION

Our results clearly demonstrate that CSI induces significant and dynamic changes in adenine nucleotide hydrolysis in the peripheral blood lymphocytes. ATP and ADP hydrolysis exhibited similar biphasic temporal profiles after the injury. In CSI animals, both hydrolyzing activities decreased at 4 h after the injury, while subsequently increasing relative to the intact control group during the rest of the recovery period. Another interesting finding was that both the CSI protocol that includes actual brain tissue damage and the sham-injury protocol

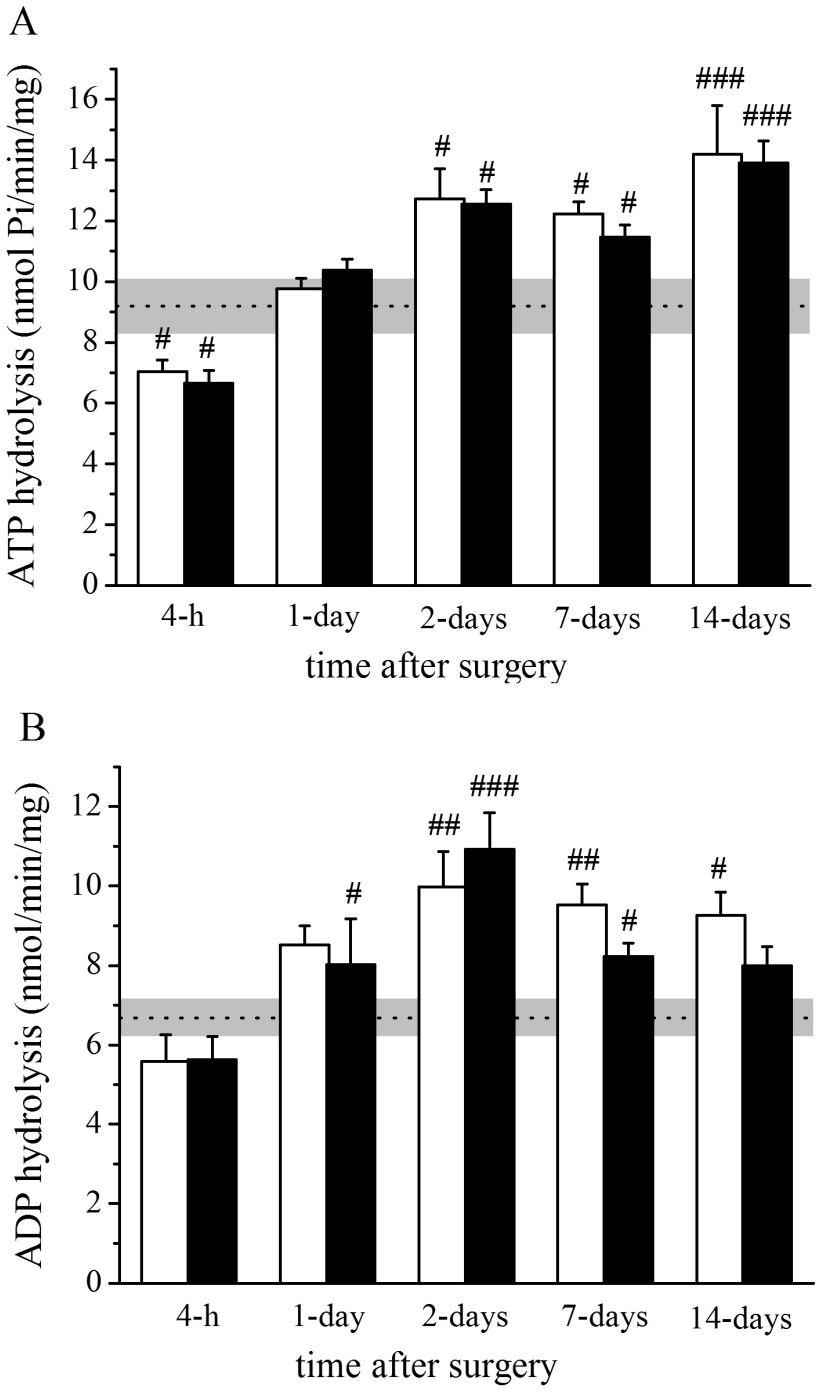

Fig. 1. ATP- (A) and ADP- (B) hydrolysis in peripheral blood lymphocyte fraction of rats at different times after CSI and sham operation. Samples were isolated from animals submitted to the CSI (dark bars) and sham operation (white bars) after 4-h, 1, 2, 7 and 14 days and specific activity is represented as mean ± SEM. Dotted lines indicate the mean value of the respective activity in the peripheral blood lymphocyte fraction of intact control animals ± SEM (gray areas). Data are mean activities (nmol Pi/min/mg) from 2 independent determinations with 5 animals / group, assayed in quintuplicate and corrected for non-enzymatic hydrolysis. Different from the intact control animals: # $p < 0.05$, ## $p < 0.005$ and ### $p < 0.001$.

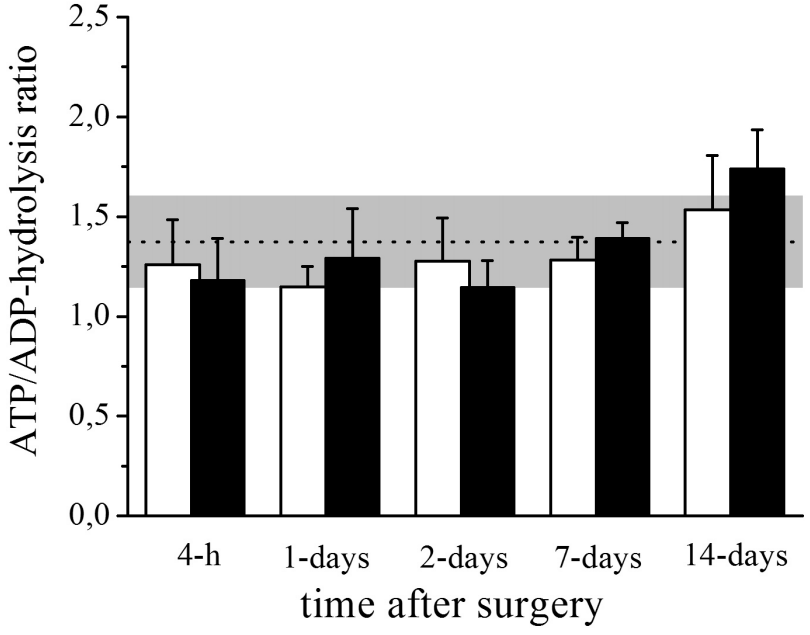

Figure 2. ATP / ADP hydrolysis of peripheral blood lymphocyte fraction ratio at different times after CSI (dark bars) and sham operation (white bars). Ratios were calculated from the data given in Fig. 1. obtained for specific ATP- (A) and ADP- (B) hydrolyzing activities after 4 h, 1, 2, 7 and 14 days from sham operation(white bars) and CSI (dark bars). Dotted lines indicate the mean value of the ATP/ADP hydrolysis ratio obtained for the intact control group ±SEM (gray area).

that includes only an incision to the skin and thin membrane covering the skull bone, induced similar changes regarding ATP and ADP hydrolysis. This finding indicates that the changes in lymphocytic ATP and ADP hydrolysis were not induced by the brain injury itself, but might be the consequence of anesthesia or some other non-specific triggers present in both experimental groups. Yet another important finding regarding lymphocytic ATP and ADP hydrolysis was that their ratios were comparable in CSI, sham-operated and intact control groups and remained unchanged throughout the recovery period. The ATP/ADP hydrolysis ratio of 1.4 ± 0.2 (Kukulski et al., 2005) indicates E-NTPDase 1 as the major contributing ectonucleotidase on the lymphocyte surface.

Considering lymphocytic AMP hydrolysis, activity was significantly increased during the whole recovery period, both in the CSI and sham groups in respect to intact animals. Since E-5NT is the major AMP hydrolyzing enzyme in the serum compartment, this finding implies that brain injury and sham operation induce upregulation of E-5NT. However, it seems that the changes obtained for AMP hydrolysis were more brain-injury specific, since at days 2 and 7 post-injury there were significant differences between the CSI and sham groups. Maximum E-5NT induction was observed at days 1 and 2 post-injury in the sham group, implying that the operation itself induced a transitory lymphocytic response, while CSI induced a sustained increase in E-5NT activity, implying that CSI induced a longer-lasting lymphocytic response.

The obtained results suggest that CSI and sham operation induce upregulation of the whole enzyme chain for adenine nucleotide hydrolysis, namely NTPDase 1 and E-5NT. Only immediately after the injury was a decrease in ATP hydrolysis observed, suggesting decreased NTPDase 1 abundance on the lymphocyte surface. It was previously demonstrated

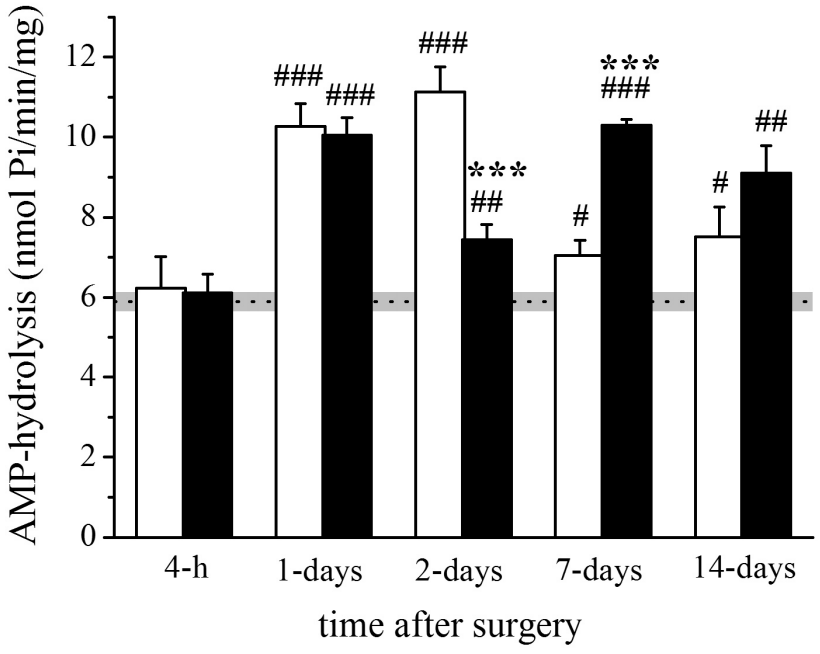

Figure 3. AMP hydrolysis in rat peripheral blood lymphocyte fraction at different times after CSI and sham operation. Samples were isolated from animals submitted to the CSI (dark bars) and sham operation (white bars) after 4 h, 1, 2, 7 and 14 days and specific activity is represented as mean ± SEM. Dotted lines indicate the mean value of the respective activity in the peripheral blood lymphocyte fraction of intact control animals ± SEM (gray areas). Data are mean activities (nmol Pi/min/mg) from 2 independent determinations with 5 animals/group, assayed in quintuplicate and corrected for non-enzymatic hydrolysis. Different from sham-operated animals, $p < 0.001$ (***). Different from the intact control animals, # $p < 0.05$, ## $p < 0.005$ and ### $p < 0.001$

that shear stress induced the removal of the NTPDase 1 enzyme protein from the surface of endothelial cells (Yegutkin et al., 2000). In the light of previous findings, we speculate that the obtained decrease of NTPDase 1 activity might be caused by a rapid shedding of the enzyme protein from the lymphocyte surface.

The upregulations of E-NTPDase1 and E-5NT were previously observed in endothelial cells (Eltzschig et al. 2003) and B-lymphocytes (Coppola et al., 2005) after hypoxia, as well as in activated lymphocytes (Leal et al., 2005; Vuaden et al., 2007). Thus, we speculate that ischemic/hypoxic conditions induced by anesthesia and surgery as the immediate causes, induced the activation of lymphocytes that was evident through increased E-NTPDase1 and E-5NT activities. The sustained increase in NTPDase 1 and E-5NT after CSI and sham operation further implies that both procedures lead to the accumulation of adenosine in the vascular compartment.

It should be noted, however, that ectonucleotidases might have additional roles other than those related to nucleotide hydrolysis (Zimmermann 2000; 2001). Ectonucleotidase shares substantial amino sequence homology and has a functional association with cell adhesion molecules (Lin et al., 1991; Dzhandzhugazyan and Bock, 1997; Salmi and Jalkanen, 2005; 2012; Bours et al., 2011) involved in the processes of adhesion and recognition between different cell types. It was previously observed that E-5NT mediates lymphocyte binding to the vascular endothelium in inflamed human skin (Arvilommi et al., 1997). In addition, engagement of lymphocytic E-5NT was shown to induce an enhanced adhesion of lymphocytes to the endothelial cells through the clustering of lymphocyte function-associated an-

tigen 1 (LFA1, CD11a-CD18) (Airas et al., 2000). Consistent with reported findings was the observation that E-5NT expression is required for the entry of T lymphocytes into the CNS (Mills et al., 2008). Regarding NTPDase1, Kansas et al., (1991) showed that the binding of anti-NTPDase 1 mouse antibody to NTPDase 1 induces rapid and strong homotypic adhesion in various human B cell lines. Taken together, our findings suggest that E-5NT and NTPDase 1, beside their enzymatic roles, might participate in lymphocytes adhesion and thereby contribute to the modulation of inflammatory processes after injury and sham operation.

In summary, our study shows that both brain injury and sham operation induce the upregulation of lymphocytic NTPDase 1 and E-5NT activities. The obtained changes suggest the activation of lymphocytes and modulation of effector cytokine production. Modulation of the peripheral immune response may further promote the progression of a secondary injury, thereby leading to the development of other neurological and non-neurological symptoms that frequently appear after brain injury. Therefore, changes in the adenine nucleotide metabolism in peripheral lymphocytes suggest that ectonucleotidases and purinergic signaling may have important roles in the course of recovery after brain injury.

Acknowledgments - This study was supported by Serbian Ministry of Science and Technology project No. III41014.

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