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EXPRESSION OF MAJOR ECTONUCLEOTIDASES AFTER CORTICAL STAB BRAIN INJURY IN RATS: A REAL-TIME PCR STUDY

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Abstract - Ectonucleotidases are cell surface-located enzymes responsible for the extracellular degradation of nucleotides. They are comprised of several protein families: ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPases) and ecto-5'-nucleotidase. Previously we showed that cortical stab injury alters ectonucleotidase activities in the rat brain, but that the specific enzymes responsible for these changes were not identified. In this study we investigated the gene expression of the specific ectonucleotidase enzymes, NTPDase1-3, NPP1-3 and ecto-5'-nucleotidase, two and seven days after cortical stab injury in rats, using real-time PCR. Two days after the injury we observed only one significant change: the downregulation in NTPDase2 mRNA expression. Our results indicate that traumatic brain injury induces significant upregulation of NTPDase1, NTPDase2 and ecto-5'-nucleotidase transcripts, and the downregulation of NPP1, seven days after the injury. Thus, traumatic brain injury has diverse impacts on ectonucleotidases gene expression, which may be reflected in the enzyme activities and extracellular nucleotide concentrations in the perilesional tissue.

Key Words: Ectonucleotidases, traumatic brain injury, NTPDase, NPP, ecto-5'-nucleotidase.

INTRODUCTION

Adenine nucleotides and nucleosides are ubiquitously present molecules. They have a central role in energy metabolism and play important roles as extracellular messengers (Burnstock, 2013). Once released in the extracellular space, the extracellular actions of nucleotides and nucleosides are mediated through two types of purinergic receptors: receptors P2 and P1. P1 are receptors for nucleosides and are widely expressed in the central nervous system (CNS), and comprise four subtypes of G protein coupled receptors (A1, A2a, A2b and A3). P2 encompasses two classes of ATP/ADP receptors: P2X which are ion channels and P2Y which are G protein coupled receptors. Several subclasses of P2X and P2Y receptors are present in the CNS (Ralevic and Burnstock, 1998).

In the nervous tissue, extracellular nucleotide concentrations are low (Burnstock et al., 2011). Different brain pathologies cause a rise in their extracellular concentrations and consequently changes in the purinergic signaling. Prolonged activation of P2 receptors may lead to inflammation, apoptosis/necrosis and proliferation (Burnstock et al., 2004; Abbracchio et al., 2009). On the other hand, adenosine is considered neuroprotective, mainly due to its

ability to limit glutamate excitatory actions by acting on presynaptic A1 receptors (Burnstock et al., 2011). Thus, the inactivation of nucleotides in the extracellular space represents an important control of neurotransmitter-mediated signaling.

Several enzyme families, often termed as ectonucleotidases, are responsible for nucleotide degradation in the extracellular space. Major ectonucleotidases include ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPases) and ecto-5'-nucleotidase (e-5NT) (Zimmermann, 1996).

E-NTPDases hydrolyze nucleoside triphosphates and diphosphates to nucleoside monophosphates. The first three (NTPDase1-3) of this eight-member family are expressed in the brain (Zimmermann, 1996). E-NPPases use a wider palette of substrates; besides nucleoside triphosphates and diphosphates, they also hydrolyze dinucleoside polyphosphates, ADP ribose and NAD^+. Of seven NPPs, at least three (NPP1-3) are present in the mammalian brain (Cognato et al., 2008). Various nucleoside monophosphates are hydrolyzed by e-5NT and this glycositol glycosylphosphatidylinositol (GPI)-bound protein is widely expressed in vertebrate brain (Zimmermann, 1992; Zimmermann et al., 1993).

Therefore, ectonucleotidases terminate P2X-mediated signaling by removing ATP, but their hydrolysis products may activate other receptor types (i.e. ADP acts on P2Y, adenosine on P1 receptors).

The specific roles of ectonucleotidases in neuropathological processes are becoming better described and recognized. Accordingly, ectonucleotidase enzyme activities and expression patterns are altered in different brain pathologies such as ischemia, epilepsy and experimental demyelination (for review see Deaglio and Robson, 2011; Amadio et al., 2011).

Our group focused on ectonucleotidase activities and protein expression after brain injury. We characterized e-5NT (Bjelobaba et al., 2011) and NTPDase3 (Bjelobaba et al., 2010) responses in a somatosensory cortical injury model. However, although we estimated changes in ATP and ADP hydrolyzing activity (Nedeljkovic et al., 2006; Bjelobaba et al., 2009), the specific enzymes responsible for these changes were not identified. The aim of this study was to estimate the changes in the mRNA expression profiles of the major ectonucleotidases, two and seven days after the stab injury in rat somatosensory cortex.

MATERIALS AND METHODS

Animals

Three-month-old male Wistar rats weighing 250 ± 30 g at the time of surgery were used in this study. The animals were housed three per cage, with free access to food and water, and were subjected to a 12 h light/dark cycle,. All animals were treated in accordance with the principles from the Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23), and the protocols were approved by the Belgrade University Animal Care and Use Committee. All efforts were made to minimize the number of used animals and their suffering.

Surgical procedure

A total of 20 animals were randomly organized into the following groups (n = 4 per group): control group (C) – intact rats; sham-operated group (S) – animals that underwent surgical procedure without skull opening, sacrificed two or seven days post-surgery (S2 and S7); lesion group (L) – animals subjected to a cortical stab injury (CSI) and were sacrificed two or seven days post injury (L2 and L7). Before starting the surgical procedure, the rats were anesthetized with 50 mg/kg Zoletil (Virbac, France). After the onset of anesthesia, the heads were shaved and the rats were placed in a stereotaxic frame. Using a sterile scalpel blade, the head was incised along the midline of the scalp to expose the bregma, and the excess of blood was collected with cotton swabs. A hand-held 1-mm-wide dental drill was inserted vertically into the cranium on the left side. The coordinates of the stab lesion to the left somatosensory cortex were 1.5 mm posterior to the bregma, 2.5 mm

lateral from the midline, and to a depth of 2 mm into the brain. The incision was closed with sutures. Rats submitted to sham injury were anesthetized, placed in the stereotaxic frame and subjected to the same surgical procedure, without causing further damage to the skull. The animals were placed in a heated room and monitored while recovering from anesthesia. Intact, age-matched animals were processed as controls.

RNA isolation and gene expression analyses

Four rats from each group were used for gene expression analysis. The animals were killed with an anesthetic overdose and transcardially perfused with cold saline. Immediately after decapitation, the brains were quickly removed from the skull. From the injured (left) cortices, 2 mm sections around the center of lesion were dissected on ice and immediately frozen in liquid nitrogen. The same size tissue explants were dissected from the left cortices of sham and intact controls. Tissue was stored at -80°C until processed using the TRIzol isolation method (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. After isolation of total RNA and treatment with DNase (Fermentas, St. Leon-Rot, Germany), RNA concentrations were measured on an Eppendorf BioPhotometer Spectrophotometer UV/VIS (Eppendorf, Wien, Austria), and the quality of RNA was checked on 1% agarose gel (Bioline, London, UK). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was conducted using SYBR Green technology (Applied Biosystems, Carlsbad, CA, USA) and analyzed on AbiPrism 7000 (Applied Biosystems, Carlsbad, CA, USA) using the following thermal profile parameters for each examined gene: 2 min at 50°C, 10 min at 95°C, 15 s at 95°C and 1 min at 60°C (the last two steps were repeated in 40 cycles).

Each reaction product was checked on agarose gels stained with ethidium bromide for specificity of amplification. Target gene expression levels were determined by the comparative 2^(-delta Ct) quantification method, using β-actin as a reference gene. Linear regressions analysis of the mean amplification Ct values showed no effects of injury on the expression of β-actin mRNA, and therefore justified the use of β-actin as a suitable reference gene for the analysis of mRNA expression in this study. Used primers (Invitrogen, Grand Island, NY, USA) were designed in the free-access internet program "Primer 3" and primer sequences were as given in Table 1. β-actin (f) 5'-agattactgccctggctcct-3', (r) 5'-acatctgctggaaggtggac-3'; NPPase1 (f) 5'-ccagaatcacatggcataattg-3', (r) 5'-cggctgtcctttgtaccaca-3'; NPPase2 (f) 5'-gacagatgtggggaagtacga-3', (r) 5'-tgcagaccacttggtagttgg-3'; NPPase3 (f) 5'-gcagaagacctttgggttga-3', (r) 5'-caaataatggtttcgaatgtgg-3'; NTPDase1 (f) 5'-cccagctgaacagccattat-3', (r) 5'-gatgaacagccctgtgatga-3'; NTPDase2 (f) 5'-ggccaaagggctactctacc, (r) 5'-gttcctgacaggctgacgat-3'; NTPDase3 (f) 5'-acggttacagcaccaccttc, (r) 5'-acagctgtgggtcaccagtt-3'; e-5NT (f) 5'-caaatctgcctctggaaagc, (r) 5'-accttccagaaggaccctgt-3'.

Statistical analysis

All data are shown as means ± SEM. Statistical significance of differences between the groups was determined using one-way analysis of variance (ANOVA for repeated measures). P values less than 0.01 (P <0.01) or 0.05 (P <0.05) were considered statistically significant.

RESULTS

Effects of CSI on ectonucleotidases gene expression

NTPDase1-3

The expression pattern of NTPDase1-3 after cortical stab injury is shown in Fig. 1. Two days after the injury, only NTPDase2 expression was significantly altered and downregulated to approximately half the level in the corresponding sham group (P <0.01, Fig. 1B). Seven days after the injury, both NTPDase1 and NTPDase2 were significantly upregulated by about 3- and 2-fold, respectively (P <0.01, Figs. 1A, B) in comparison to the corresponding sham controls.

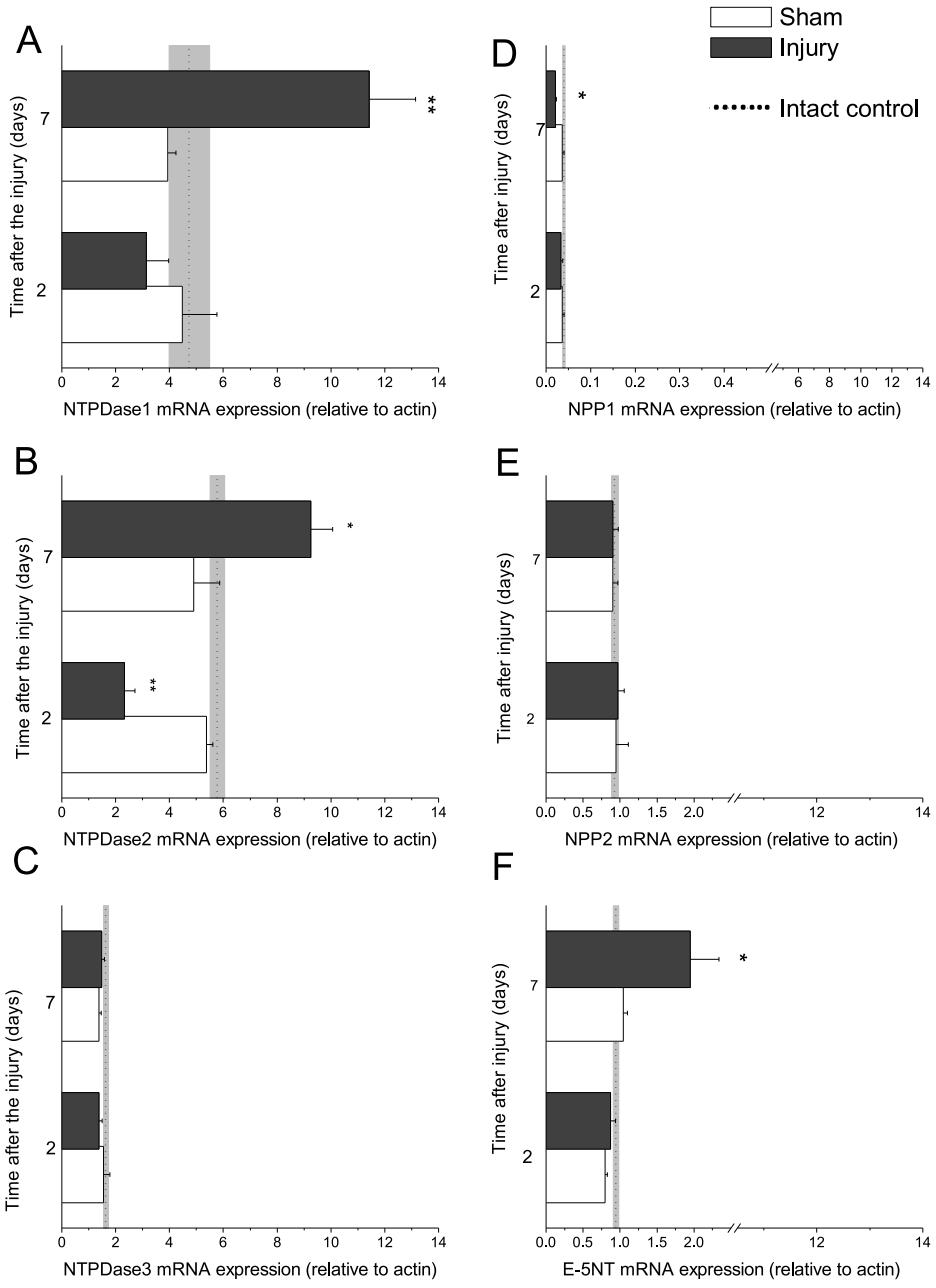

Fig. 1. Effect of CSI on ectonucleotidases mRNA expression. Expression of ectonucleotidases mRNA was measured by RTQ-PCR. The levels of each mRNA were normalized to those of the housekeeping gene β-actin. Ectonucleotidases mRNA relative expression values were compared to the corresponding control group and presented as mean ± SEM. **A-C.** The expression profile of NTPDase1-3 mRNA after the CSI. **(A)** NTPDase1 mRNA expression is upregulated ~2 fold at seven days after the injury, when compared to sham control ($P < 0.01$). **(B)** CSI induces a biphasic response in NTPDase2 mRNA expression: significant downregulation at two days after the injury and almost 3-fold upregulation seven days post-injury in comparison to corresponding sham controls ($P < 0.01$). **(C)** The levels of NTPDase 3 mRNA remained unchanged after CSI. **(D, E)** The expression profile of NPP1-3 mRNA after the CSI. **(D)** CSI has no impact on NPP1 mRNA expression at two days after the injury; it was 1.8-fold downregulated at seven days post-injury ($P < 0.05$). **(E)** NPP2 mRNA expression was not changed after CSI during the time course of study. **(F)** The level of e-5NT mRNA was not affected by CSI at two days post-injury; it was upregulated at seven days, when matched to corresponding sham controls.

NTPDase3 mRNA expression did not change significantly (Fig. 1C).

NPP1-3

NPP1 and NPP2 mRNA expression profiles are shown in Figs. 1D and 1E. NPP3 expression was very low and did not change in response to the injury (data not shown). NPP1 mRNA expression (Fig. 1D) was not affected by CSI after two days; seven days after the injury it was decreased to about half the level detected in the corresponding sham group ($P < 0.05$). In contrast, NPP2 mRNA expression remained unchanged after the injury (Fig. 1E).

Ecto-5-nucleotidase

The effect of CSI on E-5NT mRNA expression is shown in Fig. 1F. Although two days after the injury E-5NT mRNA expression was not changed, it was significantly (by about 2 fold, $P < 0.01$) upregulated after seven days in comparison to the corresponding sham group.

Transcript Abundance

According to expression relative to β-actin, the most abundant ectonucleotidase transcripts in rat cortical tissue are the transcripts for NTPDase1 and NTPDase2, while the rest of the examined ectonucleotidases seem to be expressed at significantly lower levels. Comparison of transcript abundance in intact rat cortical tissue yields the sequence in the order NTPDase2 ≈ NTPDase1 >NTPDase3 > e-5NT ≥ NPP2 > NPP1 > NPP3.

DISCUSSION

This study was designed to investigate the expression profiles of major ectonucleotidases after cortical brain injury in rat, and is a continuation of our previous work on ectonucleotidases in this injury model. We showed that the rates of ATP and ADP hydrolysis follow a biphasic pattern, with initial downregulation in the first 24 hours after the injury, and significant upregulation 15 days after injury (Nedeljkovic et al., 2006). We hypothesized that the cause for the increase in ATPase/ADPase activity might be due to the upregulation of NTPDase1 at activated microglial cells (Nedeljkovic et al., 2006). Here we show that NTPDase2 may also contribute to the observed changes in nucleotides hydrolysis, at least for ATP hydrolysis. According to the results presented herein, transcripts for NTPDase1 and NTPDase2 are most abundant in the intact rat cortical tissue. Moreover, NTPDase2 mRNA displays a biphasic change in expression, with a significant downregulation two days and pronounced upregulation seven days after the injury. Interestingly, the time points selected for this study correspond to the onset (two days) and the peak of astrogliosis, and while NTPDase1 is associated with microglia (Braun et al., 2000), NTPDase2 is usually assigned to astrocytes (Wink et al., 2006). It should be noted that implantation of NTPDase2-overexpressing C6 glioma cells has dramatic impact on tumor size increase and malignancy. Therefore, it would be interesting to investigate the involvement of NTPDase2 in astrogliosis of different etiologies.

On the other hand, the expression of NTPDase3 does not seem to be altered by the injury. Our previous findings of NTPDase3 low protein expression and absence of injury-induced changes in protein levels (Bjelobaba et al., 2010) also implied that this enzyme does not contribute much to the observed alterations of ATP/ADP hydrolyzing activities after stab brain injury in rats (Nedeljkovic et al., 2006).

Compared to other ectonucleotidases, NPPases have been less thoroughly investigated in the CNS and related pathologies. We have investigated NPP1 protein distribution by immunohistochemistry, and in the rat cortex this enzyme seems to be expressed by neurons (Bjelobaba et al., 2006). In this study, we found that NPP1 gene expression is downregulated at seven days post-injury, which could be a reflection of ongoing neuronal degeneration.

Surprisingly, we found NPP2 mRNA levels to be stable after injury. A study conducted after hippocampal brain injury in rats, at similarly chosen time points (2 and 5 days), showed striking upregulation of

NPP2 protein in white matter astrocytes (Savaskan et al., 2007). However, the tissue sampled for our analyses did not contain any white matter, since in our model of stab injury, damage of white matter does not occur. Our results indicate that NPP2 transcript is the most abundant among the investigated NPPs, although NPP2 protein expression in the rat cortex seems to be very restricted and confined to layer IV (Savaskan et al., 2007).

It was already shown that the transcript of NPP3 is present in cortical tissue and in astrocytes of the developing rat brain (Cognato et al., 2008), but based on low level mRNA expression in real-time PCR analyses, we conclude that NPP3 does not contribute significantly to the control of extracellular nucleotide levels.

We speculate that the role of NPP enzymes might be more region-specific. For instance, NPP1 enzyme exhibits the highest activity in the cerebellum, hypothalamus and hippocampus (Asensio et al., 2007), while NPP2 distribution is confined to white matter regions (Savaskan et al., 2007).

In our previously published papers, we have already addressed e-5NT enzyme activity and protein expression in detail (Nedeljkovic et al., 2006; Bjelobaba et al., 2011). It should be noted that the observed mRNA expression profile of e-5NT corresponds well to the protein expression with near to control levels 2 days after the injury and an increase seen at seven days post-injury. Nevertheless, the abundance of e-5NT mRNA is several times lower when compared to NTPDases1 and NTPDases 2 (Nedeljkovic et al., 2006). Based on immunohistochemical findings of astrocytic induction of e-5NT, upregulation in gene expression at seven days post-injury may also be assigned to astrocytes.

In conclusion, our results suggest that the major ectonucleotidases are differently challenged by cortical stab injury. NTPDase2 and NTPDase1 have the highest capacity for ATP hydrolysis, in physiological as well as pathological conditions. NPP enzymes have a lower basal expression, but while the expression of NPP2 is stable, injury induces a downregulation of NPP1. It seems plausible that injury-induced upregulation of NTPDases and e-5NT transcripts is due to astro/microgliosis and that these cells are more involved in regulating extracellular nucleotide/nucleoside levels in pathological conditions.

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