Regional changes in ectonucleotidase activity after cortical stab injury in rat

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Abstract. During a variety of insults to the brain adenine nucleotides are released in large quantities from damaged cells, triggering local cellular and biochemical responses to injury. Different models of brain injury reveal that the local increase in adenine nucleotides levels is followed by a compensatory up-regulation of ectonucleotidase enzymes that catalyze sequential hydrolysis of ATP to ADP, AMP and adenosine. However, recent studies imply that changes in adenine nucleotides release may also occur in the areas distant from the site of direct damage. Therefore, in the present study we have used the model of cortical stab injury to analyze extracellular ATP, ADP and AMP hydrolysis in the membrane preparations obtained from the brain regions that were not subjected to direct tissue damage. The brain regions analyzed were contralateral cortex, hippocampus, caudate nucleus, thalamus and hypothalamus. It was evidenced that cortical stab injury induced early widespread decrease in AMP hydrolysis in all brain areas tested, except in the hypothalamus, without changes in ATP hydrolysis. These findings imply that brain injury affects global extracellular adenine nucleotide and nucleoside levels, consequently affecting neuronal function in the regions distant to the primary damage.

Key words: Ectonucleotidase — Ecto-5'-nucleotidase — ATP — AMP — Adenosine — Stab injury — Brain — Rat

Introduction

Brain injury induces release of wide variety of mediators that influence local cellular and biochemical responses to injury. One such mediator is ATP which is massively released from damaged cells reaching high concentration in the extracellular space (Juranyi et al. 1999; Melani et al. 2005; Franke et al. 2006). By acting at specific purinergic P2 receptors, ATP exhibits both tissue protective (Rathbone et al. 1992b; Franke et al. 1999; Davalos et al. 2005) and destructive effects (Volonte et al. 1999; Amadio et al. 2002; Franke et al. 2006). Differential effects of ATP are likely mediated by the levels of ligand produced and the specific pattern of P2 receptors expression on target cells.

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Cellular responses induced by extracellular ATP are terminated by the action of ectonucleotidase enzymes. E-NTPDases (ecto-nucleotide triphospho-diphosphohydrolases; CD39), degrade ATP to ADP or directly to AMP, whereas ecto-5'-nucleotidase (CD73) converts AMP to adenosine (Zimmermann 2000). Adenosine is potent neuromodulator and neurotransmitter, acting at its own G-protein coupled P1 receptor family (Ralevic and Burnstock 1998) and eliciting diverse effects on neuronal excitability and neurotransmitter release (Jacobson et al. 1999).

It is well known that brain injury and massive ATP release induce compensatory up-regulation of ectonucleotidase enzymes. Up-regulation of ectonucleotidase activities in damaged tissue was reported in different models of epilepsy (Nagy et al. 1997; Bonan et al. 2000a,b) and ischemia (Braun et al. 1998; Villa et al. 2002). We have recently described temporal changes in ectonucleotidase activities in the damaged tissue in the model of cortical stab injury (CSI). In the model, ATP hydrolysis catalyzed by E-NTPDase was significantly

up-regulated 14 days after the injury (Nedeljkovic et al. 2006), whereas AMP hydrolysis, catalyzed by ecto-5'-nucleotidase changed in a biphasic manner, with an immediate early decrease that lasted four hours after the injury (Nedeljkovic et al. 2008), followed by a prominent increase, that persisted two weeks after the injury (Nedeljkovic et al. 2006).

Results of recent studies imply that brain injury induces global changes in brain energy metabolism, which first results in loss of ionic gradients with consequent depolarization. Under these conditions there is depletion of intracellular ATP content (Manville et al. 2007), that could be due to either intracellular degradation of ATP (Latini and Pedata 2001) or its release to the extracellular space (Melani et al. 2005). Therefore in the present study we have used the model of CSI to analyze extracellular ATP and AMP hydrolysis in the distant brain regions that were not directly affected by tissue damage.

Materials and Methods

Animals

The study was performed on 3-month-old male rats of the Wistar strain (250–350 g body weight at the time of surgery). Animals were subjected to 12-h light/dark cycle, housed 3/cage, with free access to food and water.

Surgery

All animals were treated in accordance with the principles from 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.

Animals were anesthetized with diethyl ether and placed in a stereotaxic frame. Scalp was shaven and incision was made along the midline of the scalp to expose bregma. CSI was performed as previously described (Nedeljkovic et al. 2006, 2008). In order to assess the effect of diethyl ether anesthesia on ectonucleotidase activity, another set of animals was submitted to the same procedure under the anesthesia except inflicting the stab injury (sham-operated animals, $n = 4$). Animals of both groups were placed in heated room and monitored while recovering from anesthesia. At 4 and 24 h after sham-operation or CSI, the animals were decapitated (Harvard apparatus) and brains were removed for immediate plasma membrane preparation. Intact age-matched animals ($n = 4$) were also processed as intact controls.

Plasma membrane preparation

After decapitation, brains were removed and the right (contralateral) cortices, hippocampi, caudate nuclei, thalami and hypothalami from each group were dissected and pooled for a preparation of plasma membranes. The preparation was obtained essentially following the procedure of Gray and Whittaker (1962) as previously described Nedeljkovic et al. (1998). Protein content was determined by the method prevously descibed by Markwell et al. (1978) and samples were kept on –70°C until use.

Enzyme assays

All enzyme activity assays were performed as described previously (Nedeljkovic et al. 2006) under the conditions of initial velocity and substrate saturation. The reaction medium used to assay ATP hydrolysis contained (in mmol/l): 50 Tris-HCl buffer (pH 7.4), 0.5 EDTA, 5 $MgCl_2$ in the final volume of 200 µl. The reaction medium used to assay ecto-5'-nucleotidase activity contained (in mmol/l): 100 Tris-HCl buffer (pH 7.4), 10 $MgCl_2$ in the final volume of 200 µl. Membrane preparations (20 µg of proteins for ATP hydrolysis and 50 µg for AMP hydrolysis) were added to the reaction mixture, pre-incubated for 10 min and incubated for 15 min (ATP hydrolysis) or 30 min (AMP hydrolysis) at 37°C. The reaction was initiated by the addition of ATP or AMP to a final concentration of 1.0 mmol and stopped by the addition of 20 µl of 3 mol/l perchloric acid. The samples were chilled on ice and were taken for the assay of released inorganic phosphate (Pi) (Pennial 1966).

Data analysis

The data obtained for ectonucleotidase activities were expressed as mean specific activities (nmol Pi/mg protein/min) ± S.E.M from $n \geq 3$ independent determinations performed in duplicate. Statistical evaluation was carried out using one-way ANOVA (Origin 7.0 software package). The value $p < 0.05$ was considered statistically significant.

Results

The aim of the present study was to analyze ATP and AMP hydrolysis in distant brain regions that were not subjected to direct brain damage. We used the model in which CSI was inflicted to the left cerebral cortex, whereas ATP and AMP hydrolysis were analyzed in the membrane preparations obtained from the contralateral cortex (Ctx), hippocampus (Hip), caudate nucleus (CN), thalamus (Th) and hypothalamus (HT), 4 and 24 h following injury.

Figure 1 present regional levels of ATP hydrolysis 4 and 24 h after the surgery. In all brain regions analyzed, sham operation did not affect ATP hydrolysis over the time. In the CSI group, the level of ATP hydrolysis remained unchanged over time in respect to both sham-operated and intact control animals.

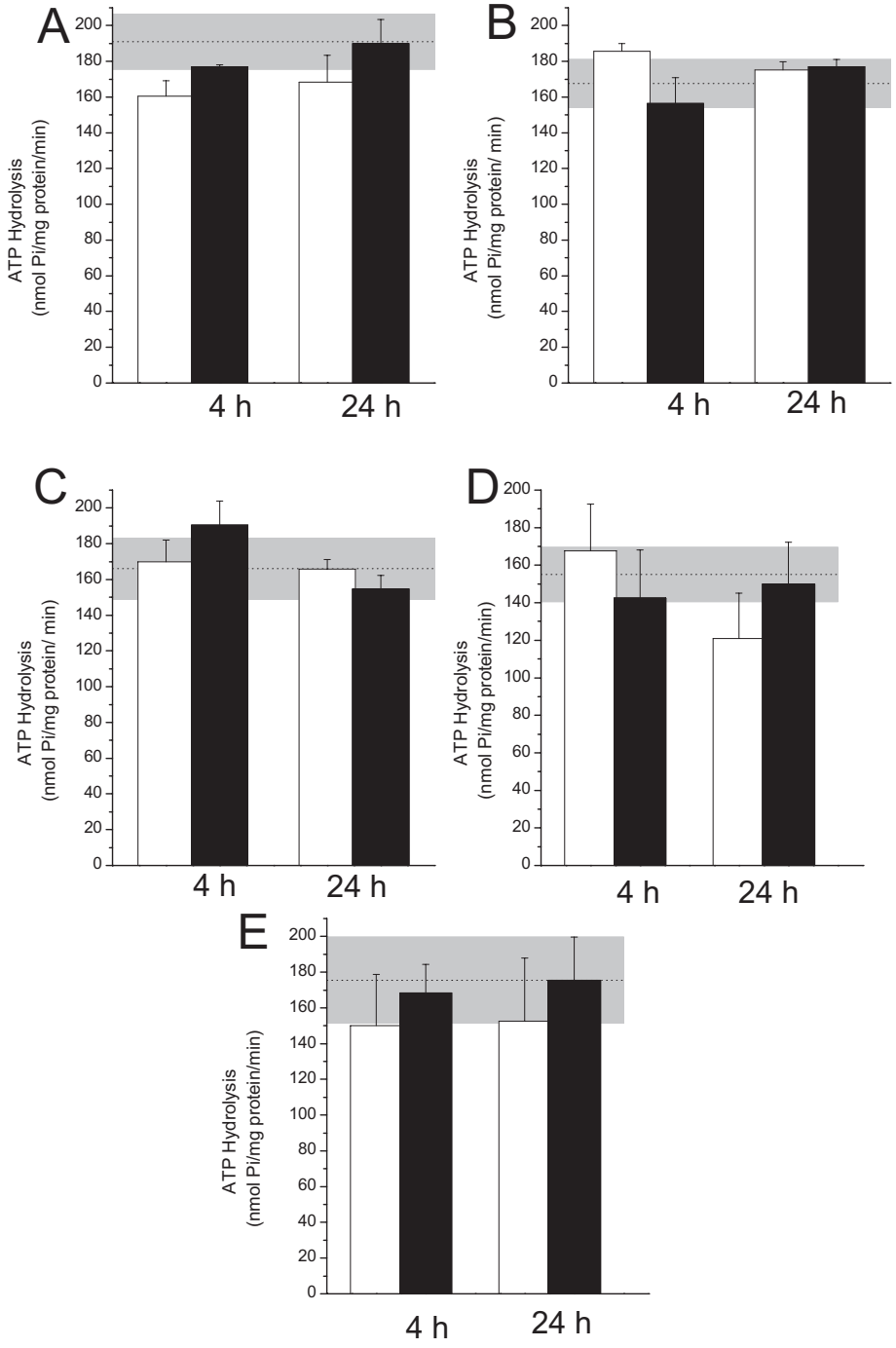

Figure 1. ATP hydrolysis (nmol Pi/mg/min) ±SEM in the plasma membrane preparations obtained from the contralateral cortex (A), hippocampus (B), caudate nucleus (C), thalamus (D) and hypothalamus (E), in sham animals (white bars) and animals submitted to CSI (black bars) 4 and 24 h after the surgery. Dot lines indicate level of ATP hydrolysis in intact control animals ±SEM (gray areas).

Figure 2 represents regional changes in AMP hydrolysis, 4 and 24 h after the sham surgery or CSI. In all brain regions analyzed, AMP hydrolysis was comparable in sham animals and in the intact controls. However, CSI induced significant decrease in AMP hydrolysis 4 h after the injury in Ctx (46.3 ± 8.7%, $p < 0.001$), Hip (27.9 ± 7.8%, $p < 0.05$) and CN (19.0 ± 6.2%, $p < 0.05$). Levels of AMP hydrolysis remained unchanged in the Th and HT.

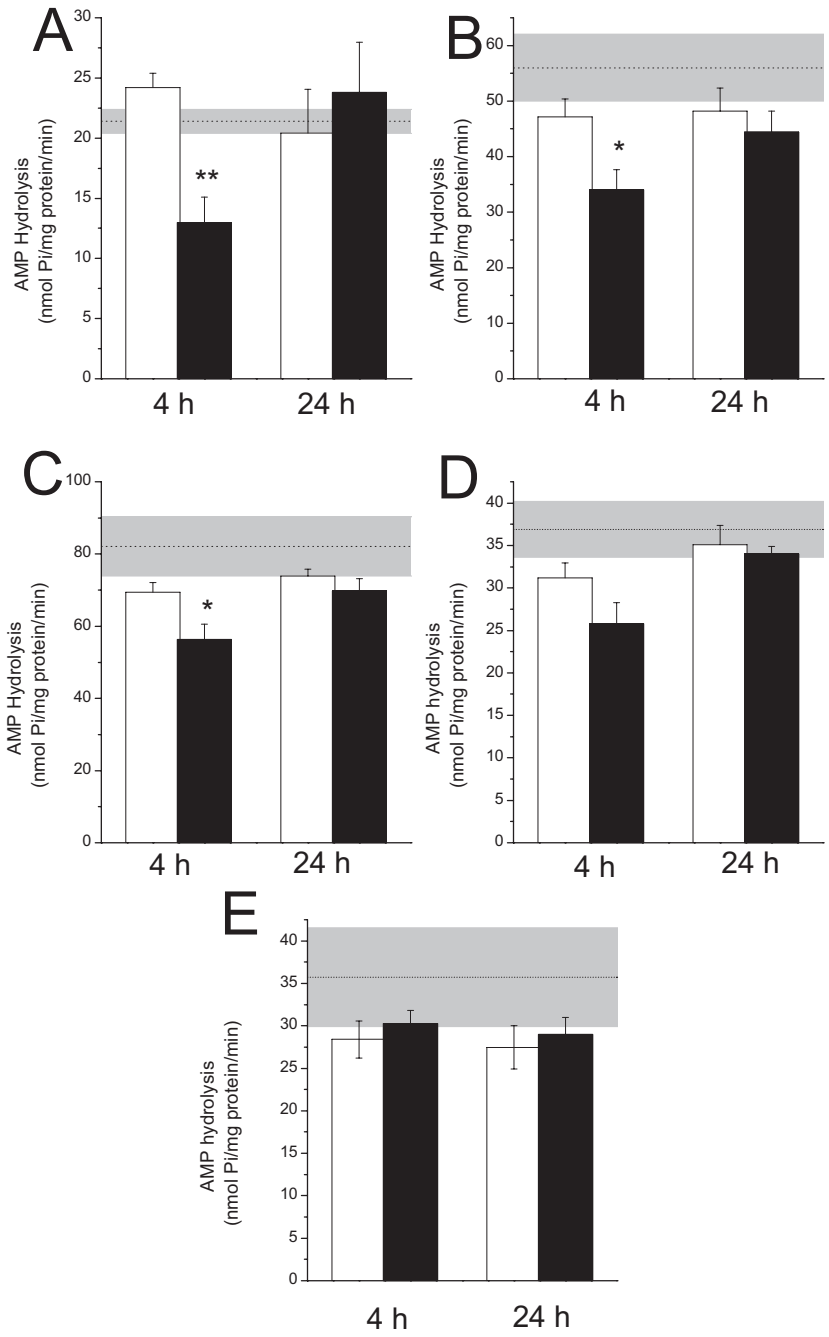

Figure 2. AMP hydrolysis (nmol Pi/mg/min) ±SEM in the plasma membrane preparations obtained from the contralateral cortex (A), hippocampus (B), caudate nucleus (C), thalamus (D) and hypothalamus (E), in sham animals (white bars) and animals submitted to CSI (black bars), 4 and 24 h after the surgery. Dot lines indicate level of AMP hydrolysis in intact control animals ±SEM (gray areas). Different from sham-operated animals, * $p < 0.05$, ** $p < 0.001$.

Discussion

It is well known that ATP induce numerous responses to injury, including early proliferation of microglia (Rathbone et al. 1992a), astrocytes (Hindley et al. 1994) and vascular endothelial cells (Rathbone et al. 1992c). Ectonucleotidase enzymes serve to terminate actions of extracellular ATP and cytotoxic effects of its massive accumulation (Volonte et al. 1999). Our previous studies scrutinized temporal changes in ectonucleotidase activities at the site of primary tissue

damage (Nedeljkovic et al. 2006, 2008). In the present study we have evaluated changes in the ectonucleotidase activities in distant cortical and subcortical brain areas in the same CSI model.

In order to isolate the effect of CSI from the effects of stress induced by anesthesia and surgical procedure, we first evaluated ATP and AMP hydrolysis in the group of animals subjected to sham operation. Results have shown that sham operation remained without effect on ATP and AMP hydrolysis in all brain regions tested.

The main finding of our study is that CSI induces early widespread decrease in AMP hydrolysis without changes in ATP hydrolysis in the cortical and subcortical regions distant to the site of direct damage. We shall discuss each in turn, along with the potential significance of altered extracellular adenine nucleotides metabolism in distant areas following injury.

Extracellular ATP is degraded by the action of several enzyme families, including E-NTPDases and ectonucleotide pyrophosphatase/phosphodiesterases, which have widespread and overlapping distribution in the rat brain (Nedeljkovic et al. 2003; Bjelobaba et al. 2006, 2007). We have previously reported that CSI induced significant up-regulation of whole enzyme chain for the extracellular hydrolysis of adenine nucleotides in remote brain areas, the effect that was observed two weeks after the injury (Nedeljkovic et al. 2006). Present study, however, revealed that during first 24 h period, CSI did not alter extracellular ATP hydrolysis, suggesting that the enzymes that hydrolyze extracellular ATP in distant regions were not affected by cortical injury.

The activity of ectonucleotidase enzymes primarily depends on the availability of their respective substrates. Extracellular ATP is thought to be released from damaged cells, reaching high concentration in the extracellular space. Accordingly, direct release of ATP in different models of brain injury has been demonstrated *in vitro* (Juranyi et al. 1999) and *in vivo* (Melani et al. 2005; Franke et al. 2006; Frenguelli et al. 2007). In the model of CSI, Franke and coworkers (2006) demonstrated immediate increase in extracellular ATP at the site of injury, which returned to its basal level within 30 min. However, the issue of extracellular ATP release in brain areas distant from the site of primary damage has not been directly examined, but our resuts revealed unaltered ATP hydrolysis during 24 h period after CSI.

On the other hand, our study revealed that CSI induced global reduction of AMP hydrolysis 4 h after the injury. Extracellular AMP hydrolysis is catalyzed by ecto-5'-nucleotidase, the enzyme widely expressed on the outer surface of different cell types throughout the brain (Bjelobaba et al. 2007; Langer et al. 2008). Observed decrease in AMP hydrolysis therefore could be linked to either decrease in ecto-5'-nucleotidase catalytic efficiency or reduction in its cell surface expression. It is known that ecto-5'-nucleotidase is competitively inhibited by extracellular ATP and ADP with K_i in the low micromolar range or below (Zimmermann 1996; Cunha 2001). However, if ATP hydrolysis sustains the basal level in distant areas following injury, as our result revealed, than catalytic inhibition of ecto-5'-nucleotidase by high ATP/ADP levels seems unlikely. Another explanation could be that injury induces secondary events in distant regions that lead to changes in the enzyme surface expression. However, since decrease in AMP hydrolysis was observed 4 h after the injury and dissapeared 24 h after the injury, transcriptional or translational down-regulation are unlikely to play a role. However, rapid time scale of the observed changes does imply another mechanism that modulates enzyme surface expression. Namely, ecto-5'-nucleotidase is a glycosylphosphatidylinositol anchored enzyme, that can be cleaved off the membrane by the action of intracellular phosphatidylinositol-specific phospholipase C (PI-PLC; Lehto and Sharom 1998, 2002). Although PI-PLC-mediated cleavage of ecto-5'-nucleotidase was evidenced in vascular endothelial cells (Kalsi et al. 2002), it remains to be seen whether the same mechanism is responsible for decrease in AMP hydrolysis in remote brain areas after CSI.

Since ectonucleotidases acts synergistically in the control of extracellular adenine nucleotides, the finding that CSI induces differential effects on ATP and AMP hydrolysis in remote areas suggests that injury alters extracellular adenine nucleotides levels and extent of their respective receptor activation. Reduction of ecto-5'-nucleotidase activity also imply accumulation of AMP in the extracellular space. This certainly affects neuronal environment, as AMP, in addition to ATP and adenosine, affects synaptic transmission and neuronal excitability (Schoen and Kreutzberg 1994). It is also noteworthy that priming P2X7 receptors, a subtype of ATP-gated receptor channel, with a very low ATP concentrations, induces dramatic increase in receptor's sensitivity to AMP (Chakfe et al. 2002) and release of interleukin-1 and TNF-α (Hide et al. 2000) from microglial cells. It was also suggested that P2X7 can even permeate AMP that could elevate intracellular AMP/ATP ratio (Menze et al. 2005). Recent discoveries suggest that increase in AMP/ATP ratio activates AMP-activated protein kinase, that is part of an ultrasensitive system, a "fuel gauge" for monitoring cellular energy stores (Hardie et al. 1999).

In summary, CSI induces early depression of AMP hydrolysis catalyzed by ecto-5'-nucleotidase without changes in ATP hydrolysis in remote cortical and subcortical areas. Implications of such alteration on neuronal function are likely to depend upon the ratio between ATP, ADP, AMP and adenosine and beneficial and damaging cellular cascades initiated by them. Since ATP and adenosine exert potent and generally opposing actions on neuronal activity and survival after injury, understanding how to tip

the balance in favor of the benefit could have clinical, as well as therapeutic importance in the treatment of brain injuries.

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