CacyBP/SIP in the rat spinal cord in norm and after transection – Influence on the phosphorylation state of ERK1/2 and p38 kinases

Ewelina Jurewicz, Krzysztof Miazga, Hanna Fabczak, Urszula Sławińska, Anna Filipek*

Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., 02-093, Warsaw, Poland

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ABSTRACT

Introduction: CacyBP/SIP is a multifunctional protein present in various mammalian tissues, among them in brain. Recently, it has been shown that CacyBP/SIP exhibits phosphatase activity towards ERK1/2 and p38 kinases.

Objectives: The aim of our study was to analyze the localization and level of CacyBP/SIP and its substrates, phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated p38 (p-p38) kinases, in an intact and transected rat spinal cord.

Methods: To achieve our goals we have performed Western blot/densitometric analysis and double immunofluorescence staining using rat spinal cord tissue, intact and after total transection at different time points.

Results: We have observed a decrease in the level of CacyBP/SIP and an increase in the level of p-ERK1/2 and of p-p38 in fragments of the spinal cord excised 1 and 3 months after transection. Moreover, immunofluorescence staining has shown that CacyBP/SIP, p-ERK1/2 or p-p38 co-localized with a neuronal marker, NeuN, and with an oligodendrocyte marker, Olig2.

Conclusion: The inverse correlation between CacyBP/SIP and p-ERK1/2 or p-p38 levels suggests that CacyBP/SIP may dephosphorylate p-ERK1/2 and p-p38 kinases and be involved in neural plasticity following spinal cord injury.

1. Introduction

Traumatic spinal cord injury (SCI) can lead to severe motor, sensory and autonomic dysfunction and, in consequence, it might be a major cause of death or lifelong disability. SCI causes a serious loss of neural cells, tissue destruction and attenuation of neuronal function. On the cellular level SCI initiates a series of signaling pathways, among them the MAPK pathway. The MAPK family consists of three major evolutionarily conserved members: ERK1/2, p38 and JNK (Zhao et al., 2019).

ERK1/2 kinase in the nervous system is critical for neuronal differentiation and may modulate neuronal survival. It has been demonstrated that this kinase plays an important role in making new connections and may contribute to neuronal plasticity in the adult central nervous system. It has been also shown that ERK1/2 can be activated/phosphorylated following neuronal injury either in the central or peripheral nervous system (Crown et al., 2006). Another MAP kinase, p38, was shown to play an essential role in the regulation of inflammatory responses, neurodegeneration and cell death. p38 is typically activated by cellular stress and proinflammatory cytokines (Kumar et al., 2003). These two kinases, ERK1/2 and p38, have been recently shown to interact with and be dephosphorylated by a novel phosphatase, CacyBP/SIP (Topolska-Woś et al., 2015, 2017; Rosińska et al., 2016). CacyBP/SIP is a protein particularly abundant in brain neurons (Jastrzębska et al., 2000). Since some reports suggest that higher phosphorylation of ERK1/2 and p38 kinase is connected with spinal cord injury (Zhang et al., 2019), in this work we have analyzed localization of a novel CacyBP/SIP phosphatase in this tissue and monitored whether the level of CacyBP/SIP, p-ERK1/2 and p-p38 changes after spinal cord transection. For that we performed Western blot/densitometry and immunofluorescence staining of spinal cord samples, intact (control) and after transection.

Abbreviations: BSA, bovine serum albumin; CacyBP/SIP, Calcyclin-binding protein/Siah-1 interacting protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ERK1/2, extracellular regulated kinase 1 and 2; MAPK, mitogen-activated protein kinase; MKPs, MAP kinase phosphatases; PBS, phosphate buffered saline; PBS-T, PBS containing 0.05% Tween 20; p38, mitogen-activated protein kinase responsive to stress stimuli; SCI, spinal cord injury; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TBS-T, tris-buffered saline containing 0.05% Tween 20.

*Corresponding author. Nencki Institute of Experimental Biology PAS, 3 Pasteur Street, 02-093, Warsaw, Poland.

E-mail address: a.filipek@nencki.edu.pl (A. Filipek).

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2. Methods

2.1. Spinal cord transection

All procedures performed in studies involving animals were in accordance with EU regulations (EU Directive, 2010/63/EU for animal experiments), and were approved by the First Local Ethics Committee in Poland. All procedures were conducted with care to minimize pain and suffering of animals. Briefly, experiments were performed on WAG (Wistar Albino Glaxo) female rats ($n = 24$) 3-month-old at the time of spinal cord injury. Complete spinal cord transection (SCI) was performed in adult rats ($n = 18$) at the Th9/10 level (Fig. 1A) under deep isoflurane (Aerrane; Baxter, Warsaw, Poland) anesthesia (5% to induce and then maintained at 2% in oxygen 0.2–0.3 l/min) and Butomidor s.c. administration (butorphanol; Richter Pharma AG, Wels, Austria; 0.05 mg/kg b.w.) as previously described (Miazga et al., 2018). To prevent the possibility of axonal regrowth through the cavity of the lesion, 1–2 mm of spinal cord tissue was aspirated using a glass pipette. Then, the muscles and fascia overlying the paravertebral muscles were closed in layers using sterile sutures, and the skin was closed with stainless steel surgical clips. After surgery, the animals received a nonsteroidal anti-inflammatory and analgesic treatment (s.c., Toludine/tolfenamic acid, Vetoquinol S.A., Magny-Vernais, France; 4 mg/kg b.w.) and antibiotics (s.c., Baytril/enrofloxacine, Bayer Animal Health GmbH, Leverkusen, Germany; 5 mg/kg b.w. and gentamicin, Biowet Pulawy, Sp. z o.o., Poland; 2 mg/kg b.w.) for the following 5–7 days.
The bladder was emptied manually twice a day until the voiding reflex was reestablished.

2.2. Preparation of tissue lysates, SDS-PAGE and Western blotting

Spinal cord tissue was collected from animals that were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (Morbital; 150 mg/kg) (Biowet Pulawy) and intracardially perfused with PBS. The spinal cords were immediately dissected and frozen by fast immersion in isopentane cooled to −80 °C and stored at −80 °C until future processing (Miazga et al., 2018). Fragments of control and transected spinal cord (3 days, n = 3; 1 month, n = 3; 3 months, n = 3 and control, n = 3) were homogenized with Polytron, four times for 30 s at 6000 rpm, in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, and protease inhibitors (Sigma Aldrich). Protein concentration was quantified according to Bradford’s procedure Bio-Rad reagents and 40 μg of protein was separated by SDS-PAGE (10%) with the use of molecular weight markers (Spectra Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific). Then, the proteins were transferred onto nitrocellulose and identified using appropriate primary antibodies: rabbit polyclonal anti-CacyBP/SIP (1:1000, non-commercial) (Filipek et al., 2002), mouse monoclonal anti-ERK1/2 (1:1000), rabbit polyclonal anti-p-ERK1/2 antibody (1:400), rabbit monoclonal anti-p38 (1:800), rabbit monoclonal anti-p-p38 (1:1000; all four from, Cell Signaling Technology) or mouse monoclonal anti-β-Actin-Peroxidase (1:15000, Sigma Aldrich). Blots were washed with the TBS-T buffer and then allowed to react with secondary antibodies, either goat anti-mouse IgG (1:15000, Jackson Immunoresearch Laboratories) or goat anti-rabbit IgG (1:10000, MP Biomedicals) conjugated to horseradish peroxidase. After three washes with TBS-T and two with TBS blots were developed with the ECL kit (Amersham Biosciences) and exposed against a RETINA X-ray film. The level of p-ERK1/2 and p-p38 was quantified relative to the level of total ERK1/2 and p38, respectively. The level of CacyBP/SIP was estimated relative to the level of β-actin as the internal standard. The intensities of protein bands were quantified using the Ingenius instrument and the Gene Tools from Syngene. Statistical data analysis was performed using the Student’s t-test. Differences between control and injured tissues were considered statistically significant if the p value was lower or equal to 0.05. The statistical significance was indicated by asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

2.3. Immunofluorescence staining

Spinal cords were collected from rats 1 month after transection (n = 3) or from control ones (n = 3). Rats were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (Morbital; 150 mg/kg) (Biowet Pulawy) and intracardially perfused, first for 2 min with saline and then for 20 min with cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After perfusion spinal cords were isolated, additionally fixed for 4 h in 4% paraformaldehyde, and cryoprotected with 30% sucrose in 0.02 M potassium phosphate buffer, pH 7.4 until complete saturation. Spinal cords were frozen in dry ice and stored at −80 °C. Micromt cut sections (14 μm thick) of fragment 2 (Fig. 1A) were used for staining. Sections were washed (3 × 10 min) in PBS and blocked for 1 h at RT in PBS containing 1.5% normal goat serum and 0.5% Triton X-100, to reduce unspecific binding. Then, sections were incubated overnight at 4 °C with the same antibodies as for Western blot: anti-CacyBP/SIP diluted 1:1600, anti-p-ERK1/2 diluted 1:200 or anti-p-p38 diluted 1:1000 in PBS containing 0.3% BSA. Sections were then washed (3 × 10 min) in PBS and incubated for 1 h at RT with secondary goat anti-rabbit antibodies conjugated with Alexa Fluor 488 (Life Technologies Corporation) diluted 1:500 in PBS containing 0.3% BSA. Next, sections were washed in PBS (3 × 10 min) and incubated for 2 h at RT with antibodies against the neuronal marker, NeuN (mouse monoconal; Sigma Aldrich) diluted 1:100, the oligodendrocyte marker, Olig2 (mouse monoconal; Sigma Aldrich) diluted 1: 200 or the astrocyte marker, GFAP (mouse monoconal; BD Biosciences) diluted 1:1000 in PBS. After washing in PBS (3 × 10 min) sections were incubated for 1 h at RT with secondary donkey anti-mouse antibodies conjugated with Alexa Fluor 555 (Life Technologies Corporation) diluted 1:500 in PBS containing 0.3% BSA. After washing in PBS (3 × 10 min) sections were mounted on slides using the Vectashield mounting medium (Vector Laboratories) and analyzed using a Zeiss AXIO Imager M2 microscope.

3. Results

At first, we have analyzed the level of CacyBP/SIP and its targets/substrates, p-ERK1/2 and p-p38 kinases, in three fragments of the control (C1, C2 and C3) spinal cord and after transection (T1, T2 and T3) (Fig. 1A) by applying Western blot/densitometric analysis. For all three spinal cord fragments in samples excised 1 month after transection we have observed changes in the level of all three proteins examined, when compared with control (Fig. 1B, C, D). In particular, a statistically significant decrease in the level of CacyBP/SIP in fragments 1 and 2 (Fig. 1B) was observed. In the case of p-ERK1/2, an increase was detected for fragment 1, while for p-p38 - for all three examined fragments (Fig. 1C, D).

When samples excised 3 months after transection were studied, a decrease in the level of CacyBP/SIP in fragments 1 and 3 (Fig. 1B) was found. In the case of p-ERK1/2, an increase was seen in fragments 1 and 3, while for p-p38 - in fragments 1 and 2 (Fig. 1C, D).

We also performed the same analysis for samples prepared from fragments of the spinal cord taken very early, that is 3 days after transection (Fig. Suppl. 1). We have found that the level of CacyBP/SIP exhibited a decreasing tendency when compared with control; its lowest and statistically significantly different level was detected for fragment 2 (Fig. Suppl. 1A). Regarding the level of p-ERK1/2 no changes were observed (Fig. Suppl. 1B) while as to p-p38, a clear increasing tendency was observed although the differences between control and transected spinal cord were not statistically significant (Fig. Suppl. 1C). Immunofluorescence staining of sections derived from fragment 2 of control (C2) and from a corresponding fragment of transected (T2) spinal cord with the use of antibodies against CacyBP/SIP, p-ERK1/2 or p-p38 confirmed the results obtained by Western blot (Fig. 2). Additionally, to check what types of cells express the proteins of interest, double immunofluorescence staining was performed. Fig. 2 (A, B, C) shows examples of motoneurons present in the ventral horn in the lumbar control spinal cord and the corresponding tissue excised after transection, in which CacyBP/SIP (Fig. 2A), p-ERK1/2 (Fig. 2B) and p-p38 (Fig. 2C) co-localize with the neuronal marker, NeuN.

Furthermore, Fig. 2 (D, E, F) demonstrates the co-localization of CacyBP/SIP (Fig. 2D), p-ERK1/2 (Fig. 2E) and p-p38 (Fig. 2F) with the oligodendrocyte marker, Olig2, in the lumbar dorsal horn. No co-localization of the proteins of interest with the astrocyte marker, GFAP, was detected in the ventral or dorsal horn of the examined tissue (not shown).

4. Discussion

It is well known that following SCI, in addition to the loss of function and sensation below the level of injury, secondary complications including uncontrolled muscle spasticity, hyperreflexia and autonomic dysreflexia also develop (Rachevsky and Kitzman, 2011). These secondary effects develop gradually after the state of areflexia and muscle weakness that immediately follow injury and are related to the chronic reorganization of the spinal cord circuitry due to neural synaptic plasticity (D’Amico et al., 2014). In this work we focused our attention on motoneurons that control muscle activity and are located in the ventral horns. Neural plasticity related to these neurons is one of the many factors responsible for spasticity that develops after SCI. Our data show
the localization of CacyBP/SIP and its targets, ERK1/2 and p-38, in motoneurons in the lumbar ventral horn that are known to undergo plastic changes related to spasticity developed after SCI. They also demonstrate the presence of these proteins in oligodendrocytes localized in the dorsal horn of the lumbar spinal cord. Oligodendrocytes support axonal sprouting that is known to be extensive in dorsal horns after SCI (Almeida and Lyons, 2017). Considering that MAPK signaling can be activated by synaptic activity related to neural plasticity following spinal cord transection (Thomas and Huganir, 2004), it can be hypothesized that in chronic changes like spasticity, hyperreflexia and autonomic dysreflexia developing after spinal cord injury, a MAPK cascade is involved.

On the other hand our results demonstrate that, at the cellular level, SCI can initiate a serious of molecular events, such as activation of p38 and ERK1/2 kinases, which in consequence results in expression of genes mediating the inflammatory responses (Luo et al., 2015). Increased activation of these kinases may occur due to their phosphorylation or due to inhibition of their dephosphorylation. Dephosphorylation of p-p38 or p-ERK1/2 is catalyzed by MAPK phosphatases, MKPs. From the perspective of the nervous system, little is known about the function of MKPs and how extracellular signals can regulate them. In fact, most studies concerning intracellular cascades activated in neuronal cells focused on signaling kinases and have always eluded the deactivation mechanisms.

Quite recently we have demonstrated that CacyBP/SIP exhibits phosphatase activity and is able to dephosphorylate p-ERK1/2 and p-p38 kinases (Topolska-Woś et al., 2015, 2017; Rosińska et al., 2016). In this work we have shown that CacyBP/SIP is present in neurons and oligodendrocytes of the spinal cord since it co-localized with a neuronal marker, NeuN, and an oligodendrocyte marker, Olig2. Notably, our earlier results showed neuronal localization of CacyBP/SIP in rat brain (Jastrzębska et al., 2000). In the present work we have also found that the level of CacyBP/SIP and its targets, p-ERK1/2 and p-p38, changes in the spinal cord after transection when compared to control tissue. More precisely, we have observed a decreased level of CacyBP/SIP and increased levels of p-ERK1/2 and p-p38. Considering that CacyBP/SIP is a phosphatase, our results are in agreement with those obtained for another phosphatase, MKP-1 (Hong et al., 2008). Namely, it was found that the level of MKP-1 in the injured spinal cord was decreased while the level of p-ERK1/2 was increased. In our case changes in the level of the analyzed proteins were observed in fragments of the spinal cord excised 1 or 3 months after transection. Also, it should be noted that more substantial changes between control and transected tissues were seen for p-p38 kinase than for p-ERK1/2 kinase.

5. Conclusions

Presence of CacyBP/SIP and its co-localization with p-ERK1/2 or p-p38 in neurons and oligodendrocytes of the spinal cord and the inverse correlation between CacyBP/SIP level and the phosphorylation state of the examined kinases, suggest that in these cells CacyBP/SIP may contribute to dephosphorylation of p-ERK1/2 and p-p38 kinases. The increase in p-ERK1/2 and p-p38 level, either due to activation of upstream kinases or, as we suggest, due to lower level of phosphatases, including CacyBP/SIP, might be important for neuroinflammation and/or neural plasticity following spinal cord injury.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104757.

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