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Matrix metalloproteinase 9 regulates cell death following pilocarpine-induced seizures in the developing brain

Yvonne Hoehna [a,1], Ortrud Uckermann [a,b,1], Hella Luksch [a,c], Vanya Stefovska [a,d], Jenny Marzahn [c], Marlen Theil [a], Tomasz Gorkiewicz [e,f], Maciej Gawlak [g], Grzegorz M. Wilczynski [g], Leszek Kaczmarek [e], Chrysanthy Ikonomidou [h,*]

[a] Department of Pediatric Neurology, Children's Hospital, Medical Faculty Carl Gustav Carus, Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany
[b] Department of Neurosurgery, Medical Faculty Carl Gustav Carus, Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany
[c] Department of Pediatrics, Children's Hospital, Medical Faculty Carl Gustav Carus, Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany
[d] Department of Anatomy, Histology and Embryology, Varna Medical University, 55 Marin Drinov Str., 9002 Varna, Bulgaria
[e] Department of Molecular and Cellular Neurobiology, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland
[f] Department of Biophysics, Warsaw University of Life Sciences SGGW, 159 Nowoursynowska St., 02-776 Warsaw, Poland
[g] Department of Neurophysiology, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland
[h] Waisman Center and Department of Neurology, University of Wisconsin, Madison, WI, USA

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ABSTRACT

Matrix metalloproteinases (MMPs) are involved in tissue repair, cell death and morphogenesis. We investigated the role of the gelatinases MMP-2 and MMP-9 in the pathogenesis of neuronal death induced by prolonged seizures in the developing brain.

Seven-day-old rats, MMP-9 knockout mice and transgenic rats overexpressing MMP-9 received intraperitoneal injections of pilocarpine, 250 mg/kg, to induce seizures. After 6–72 h pups were sacrificed, tissue from different brain regions was isolated and expression of MMP-9 mRNA and protein was analyzed by real-time PCR or Western blot. Additionally, brains were fixed and processed for TUNEL-staining, immunohistochemistry and in situ zymography.

We found increased numbers of TUNEL-positive cells 24 h after pilocarpine-induced seizures, most pronounced in cortical areas and the dentate gyrus, and less pronounced in thalamus. At 6–24 h, MMP-9 mRNA levels showed significant elevation compared to sham-treated controls; this effect resolved by 48 h, whereas MMP-2 mRNA levels remained stable. Cortical gelatinolytic activity, monitored by in situ zymography, was enhanced following pilocarpine-induced seizures.

The MMP inhibitor GM 6001 ameliorated cell death following pilocarpine-induced seizures in infant rats. MMP-9 knockout mice were less susceptible to seizure-induced brain injury. Transgenic rats overexpressing MMP-9 were equally susceptible to seizure-induced brain injury as wild type rats.

Our results suggest a significant contribution of MMP-9 to cell death after pilocarpine-induced seizures in the developing brain. As indicated by Western blot analysis, MMP-9 activation may be linked to activation of the Erk/CREB-pathway. The findings implicate involvement of MMP-9 in the pathophysiology of brain injury following seizures in the developing brain.

Introduction

Matrix metalloproteinases (MMPs), a family of extracellular soluble or membrane bound neutral proteases, have complex functions under normal and pathological conditions (Rivera et al., 2010; Yong, 2005; Yong et al., 1998). They are important for normal development, and are involved in remodeling of extracellular matrix and wound healing. Accumulating evidence has extended the role of MMPs into the normal physiological functions of the brain. Among MMPs, MMP-2 and MMP-9 are most abundantly expressed in the developing brain. MMP-9 is highly expressed in neuronal cell bodies and dendrites (Szklarczyk et al., 2002). Growing data suggest the association of MMP-9 with dendritic spine remodeling, synaptic plasticity, learning and memory formation (Michaluk et al., 2011; Nagy et al., 2006; Wang et al., 2008).

MMP-9 is required for late-phase long term potentiation (LTP) in hippocampus (Nagy et al., 2006) and prefrontal cortex (Okulski et al., 2007) and for spatial and emotional learning (Nagy et al., 2006).

* Corresponding author at: Department of Neurology, University of Wisconsin, 1685 Highland Avenue, Centennial Building, Madison, WI 53705, USA. Fax: +1 608 2630412.
E-mail address: ikonomidou@neurology.wisc.edu (C. Ikonomidou).
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[1] These authors contributed equally to this study.

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Bozdagi et al. (2007) and Tian et al. (2007) demonstrated that LTP is associated with significant increases in levels of MMP-9, this effect being dependent upon N-methyl-D-aspartate (NMDA) receptor activation. MMP-9 appears to exert its action directly at the level of dendritic spines (Konopacki et al., 2007; Michaluk et al., 2011; Tian et al., 2007; Wilczynski et al., 2008), possibly via cleavage of synapse associated molecules such as dystroglycan (Michaluk et al., 2009) and intracellular adhesion molecule 5 (ICAM-5) (Tian et al., 2007).

Evidence implicates involvement of MMPs in epilepsy and epileptogenesis in the adult brain (Lukasiuk et al., 2011). Activation of MMP-2 and MMP-9 occurs in the brain in the kainic acid rat seizure model (Zhang et al., 2000). In the adult rat pilocarpine seizure model, MMP-9 induces apoptotic cell death by interrupting integrin-mediated survival signaling (Kim et al., 2009). A study linking MMP-9 to epileptogenesis was published by Wilczynski et al. (2008). MMP-9 was found to promote epileptogenesis in kainate-evoked and pentylenetetrazole kindling induced epilepsy in rats and mice (Wilczynski et al., 2008). In two animal models of temporal lobe epilepsy, the kainic acid model and the pentylenetetrazole kindling model, these authors demonstrated decreased sensitivity in MMP-9 knockout mice but increased sensitivity in transgenic rats overexpressing MMP-9.

So far, no experimental data pertaining to the role of MMPs in seizures, seizure-induced injury and epileptogenesis in the developing brain exist. Our goal was to start exploring links between MMPs and seizures in the neonatal brain. Here we study whether MMPs are activated in the context of epileptic events in the developing brain and search for potential involvement of MMP-2 and MMP-9 in seizure-induced neuronal death. We used P7 rats to model neonates and the pilocarpine seizure model to model seizures. Cavalheiro et al. (1987) provided detailed behavioral and electrophysiological characterization of the pilocarpine seizure model in developing rats. On P7 pilocarpine (100–380 mg/kg) has been shown to cause behavioral changes consisting of hyperactivity, followed by hypoactivity, head and whole body tremor, loss of righting reflex, masticatory automatisms and clonic limb movements. These behavioral events correlate with electrographic changes which consist of background disorganization, appearance of cortical and hippocampal spikes which develop within minutes after injection of pilocarpine, progressively increase in frequency, evolve into continuous spikes and polyspikes and slowly resolve with return to baseline by 4 h after the injection of pilocarpine.

We studied the expression of MMP-2 and MMP-9 in the brains of P7 infant rats who developed seizures after systemic injection of pilocarpine and evaluated the effect of the MMP inhibitor GM6001 on the severity of seizure-induced neuronal death. Neurodegeneration following seizures was also investigated in MMP-9 knockout mice and MMP-9 overexpressing transgenic rats. Our findings indicate that MMP-9 is involved in the pathogenesis of seizure-induced brain injury in the developing brain.

Materials and methods

Animal model

All animal experiments were performed in accordance with the guidelines of the Technical University in Dresden, Germany and according to the rules established by the Ethical Committee on Animal Research of the Nencki Institute, based on national laws that are in full agreement with the European Union directive on animal experimentation.

Experiments were performed on 7 day old Wistar rats (Charles River Laboratories, Germany GmbH, Sulzfeld Germany), MMP-9 overexpressing Wistar rats (Wilczynski et al., 2008) and MMP-9 KO mice C57BL/6 strain (provided by Z. Werb, University of California, San Francisco, San Francisco, CA; Vu et al., 1998).

Postnatal rat pups or MMP-9 knockout mice were injected i.p. with N-methyl scopolamine (1 mg/kg bodyweight, Sigma, St Louis, MO USA) followed by pilocarpine (250 mg/kg bodyweight, Sigma, St Louis, MO, USA) after 30 min. After 2.5 h seizures were stopped by injecting diazepam (Faustan®, 1 mg/kg bodyweight in 0.9% NaCl). Saline treated littermates served as controls. The metalloproteinase blocker GM6001 was dissolved in 0.2% DMSO and administered in two doses of 40 mg/kg bodyweight each (one simultaneously with scopolamine, one simultaneously with diazepam), leading to the final dose of 80 mg/kg.

Tissue processing

At 6, 12, 24, 48 or 72 h after injection of pilocarpine animals were euthanized. For molecular biology studies tissue from parietal/frontal cortex, hippocampus and thalamus was isolated and frozen in liquid nitrogen. Tissue samples were stored at −80 °C upon isolation of RNA or protein.

The extent of brain injury was histologically characterized 24 h after injection of drugs. The animals received an overdose of chloral hydrate and were transcardially perfused with cooled 0.01 M PBS, pH 7.4 containing 1 U/ml heparin followed by perfusion with 4% paraformaldehyde in PBS. Brains were postfixed for 3–4 days in 4% paraformaldehyde at 4 °C. Knockout mice and transgenic rats were not perfused. Whole brains were removed and fixed in 4% paraformaldehyde for 3–4 days at 4 °C. Brains were embedded in paraffin and frontal sections of 10 μm thickness were prepared.

For in situ zymography, embedding in polyester wax was performed according to Gawlak et al. (2009). Whole brains were fixed in methanol–ethanol (1:3) for 24 h at 4 °C, cut into 5 mm pieces and embedded in polyester wax. Embedded brains were stored at −20 °C to preserve enzymatic activity within the tissue.

Isolation of protein

Protein was isolated from different brain regions using RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.5% Natriumdeoxycholat, 150 mM Natriumchlorid, 1 mM EDTA, 1 mM EGTA, 1 mM Natriumorthovanadat, 20 mM Natriumfluorid 0.1% SDS) supplemented with one protease inhibitor cocktail tablet (complete, Hoffmann-La Roche AG, Switzerland) in 50 ml RIPA buffer. After homogenization of the tissue and centrifugation at 12,000 ×g for 5 min the supernatants were collected. Protein concentration was determined using MicroBC assay Protein Quantification Kit (Interchim, Montluçon Cedex, France).

Western blot

Protein samples were diluted to equal concentrations and 20 μg of protein was mixed with Laemmli buffer (0.1% bromophenol blue, 50% glycerine, 1% β-mercaptoethanol, 2% SDS, 0.125 M Tris–HCl, pH 6.8) in a ratio of 4:1 and heat denaturated. Samples and a protein standard (Precision Plus protein standards dual color, Bio-Rad, Munich, Germany) were loaded on a 10% SDS gel. The samples were electrophoretically separated and transferred to PVDF membranes (0.45 μm Millipore) using the Trans Blot SD (Bio-Rad, transfer buffer: 39 mM glycine, 48 mM Tris, 20% methanol, 0.004% SDS). Membranes were washed in Tris-buffer containing 0.1% Tween 20 (TBS-T) and incubated 2×30 min in Rotiblock supplemented with 0.1% Tween 20 (Roth, Karlsruhe, Germany) to block unspecific binding sites. Subsequently the first antibody was applied over night at 4 °C. After washing (5×5 min in TBS-T) membranes were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibodies. Washing 5×5 min in TBS-T was followed by 1 min incubation in enhanced chemiluminescence solution and detection of chemiluminescence was performed on film (Hyperfilm ECL, Amersham Biosciences). Afterward, the membranes were incubated in stripping