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Plasma levels of neuron specific enolase quantify the extent of neuronal injury in murine models of ischemic stroke and multiple sclerosis

Mathias Gelderblom [a,1], Tristan Daehn [a,1], Benjamin Schattling [b], Peter Ludewig [a], Christian Bernreuther [c], Priyadharshini Arunachalam [a], Jakob Matschke [c], Markus Glatzel [c], Christian Gerloff [a], Manuel A. Friese [b], Tim Magnus [a,*]

[a] Department of Neurology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
[b] Center for Molecular Neurobiology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
[c] Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany

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ABSTRACT

Objective: We aimed at validating a plasma biomarker for neuronal damage that can be used in acute and chronic models of neurological diseases.
Methods: We investigated two different models, middle cerebral artery occlusion followed by reperfusion and $MOG_{35–55}$-induced experimental autoimmune encephalomyelitis (EAE). In stroke experiments we measured infarct sizes by magnetic resonance imaging and vital stainings and correlated them with plasma levels of neuron specific enolase (NSE) at different time points after reperfusion. Equally, in EAE experiments, we correlated NSE levels with neurological scores and histopathological damage of axons at different time points. We detected plasma NSE levels by ELISA.
Results: Plasma NSE levels correlated significantly with stroke size, EAE score and histopathological damage in EAE. Investigations into the dynamics of neuronal loss over time correlated well with the dynamics of NSE levels. NSE even predicted the onset of EAE, before clinical signs were recordable.
Conclusions: Plasma NSE is a valid and simple experimental biomarker that allows quantifying the degree of neuronal injury in a non-invasive approach.

Introduction

Non-invasively quantifying neuronal injury remains a challenge in neurological diseases. Functional scores and imaging are often crude and can only determine a rough estimate of neuronal loss in vivo. Post-mortem, neuronal cell death can be identified by a variety of immunohistochemical or biochemical approaches (Zille et al., 2012), which provide a precise estimate of the neuronal damage, correlate directly with the amount of neuronal cell death and allow the direct analysis of underlying molecular mechanisms. However, these techniques can be applied only once, the animal needs to be sacrificed and each technique demands its own tissue preparation, precluding the application of different methods in parallel. Neither can these methods be used in intra-individual longitudinal analyses during neuroprotective studies.

To overcome these drawbacks, additional surrogate markers such as serum proteins, radiological, nuclear and optical imaging techniques, might be useful to estimate the extent of neuronal damage in animal models. Compared to imaging techniques, blood markers can be readily obtained and easily quantified. Besides the amyloid precursor protein (APP) and the tau-protein one potential candidate as blood biomarker of neuronal damage is the neuron-specific enolase (NSE) (Ahmad et al., 2012).

NSE is an isoenzyme of the glycolytic enzyme enolase which is exclusively expressed in neurons and cells of neuroendocrine origin (Marangos et al., 1979). Furthermore, it was shown to be stable in biological fluids (Steinberg et al., 1983) and represents about 1.5% of total soluble brain proteins (Marangos et al., 1979). In early stages of traumatic and hypoxic brain injury NSE is frequently used to estimate prognosis (Anand and Stead, 2005).

Here we investigated whether the plasma concentration of NSE can be used to quantify the extent of neuronal injury in animal models of diseases affecting the central nervous system (CNS). We employed two exemplary models, middle cerebral artery occlusion (MCAO) and experimental autoimmune encephalomyelitis (EAE), in which neuronal damage can be precisely determined by morphological approaches and correlated with plasma NSE levels at different stages of the diseases.

* Corresponding author. Fax: +49 40 7410 55591.
E-mail address: t.magnus@uke.de (T. Magnus).
Available online on ScienceDirect (www.sciencedirect.com).
[1] Equally contributing first authors.

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Material and methods

Animals

All animal experiments were approved by the local animal licensing committee (Behörde für Lebensmittelsicherheit und Veterinärwesen), were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 83-123, revised 1996), and were performed in accordance with the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA).

In vivo stroke model

All mice were randomized and the scientists performing the experiments blinded. Temporary middle cerebral artery occlusion (MCAO) was done as previously described (Gelderblom et al., 2012). Briefly, MCAO was achieved by using the intraluminal filament method (6-0 nylon). To obtain infarct sizes of different extents animals underwent occlusion for 30 min, 45 min, 60 min and 75 min. All mice (20 to 25 g, 12 weeks) were anesthetized (isoflurane 1% to 2% v/v oxygen) and underwent analgesia (buprenorphine 0.03 mg/kg body weight intraperitoneally every 12 h for 24 h). All mice were monitored for blood pressure, heart rate, body temperature, and cerebral blood flow using transcranial temporal laser Doppler. After stroke induction, every mouse was repeatedly scored on a scale from 0 to 5 (0 no deficit, 1 preferential turning, 2 circling, 3 longitudinal rolling, 4 no movement, 5 death) immediately after reawakening and every day until sacrifice. The cerebral blood flow in the area of the MCA showed a reduction of ~90%, which did not differ between groups (data not shown). Mice were sacrificed one, three, five or seven days after reperfusion using isoflurane and decapitation. Only mice with a score greater or equal than one after reawakening were included for stroke size analysis.

Experimental autoimmune encephalomyelitis

Mice were immunized subcutaneously with 200 μg myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}; Schafer-N, Copenhagen, Denmark) in complete Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* (H37Ra, both Difco). Mice were injected with 200 ng pertussis toxin (Calbiochem) intravenously on the day of immunization and 48 h later. Mice were sex- and age-matched (6–10 weeks) and scored daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and fore limb paresis; and 5, pre-morbid or dead. Animals with a score ≥ 4 were sacrificed.

Analysis of infarct size by TCC staining

For analysis of infarct size, brains were harvested, cut into 1 mm slices (Braintree Scientific, 1 mm) and vital staining using 2% (w/v) 2,3,5-triphenyl-2-hydroxy-tetrazolium chloride (TTC, Sigma, Saint Louis, Missouri, USA) in phosphate buffer was performed. Infarct volumes were determined by blinded examiners using NIH ImageJ.

Stroke assessment by magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed repeatedly three days after stroke on a dedicated 7 T MR small animal imaging system (ClinScan, Bruker, Ettlingen, Germany) with a 4 element phased array mouse brain receiver coil and a circular polarized whole body transmit coil. The image protocol comprised T2 weighted sequence to assess localisation and extent of infarction. T2 weighted TSE sequence parameters were: TE = 57 ms, TR = 4650 ms, BW = 100 Hz/pixel, turbo factor 7, matrix = 256 × 192, FOV = 20 × 15 mm^2, 28 slices, and 0.4 mm slice thickness with 0.1 mm gap. Calculation of edema corrected stroke volumes, and non-invasive quantification of brain edema was performed as described elsewhere (Gerriets et al., 2009).

Blood samples

Following stroke, whole blood was collected by cardiac puncture with subsequent sacrificing of mice. Blood plasma was prepared by adding Ca^{2+}-EDTA, followed by centrifugation at 5000 rpm for 5 min and collection of the supernatant. Samples were immediately stored at −80 °C. In EAE experiments whole blood was collected by puncturing the submandibular vein and processed as described above.

Immunohistochemical analysis of amyloid precursor protein (APP)

For immunohistochemical analysis of spinal cords, animals were perfused with 4% paraformaldehyde (PFA; Carl Roth). Spinal cords were excised and kept in PFA for at least 48 h, dehydrated and embedded in paraffin. 3 μm thick horizontal sections were cut on a microtome and were stained following standard immunohistochemistry procedures using the Ventana Benchmark XT machine (Ventana, Tucson, Arizona, USA) with mouse monoclonal antibodies against amyloid precursor protein (APP, clone 22C11; 1:3000, Millipore, Schwalbach, Germany). For detection of primary antibodies, "Ultraview Universal HRP Multimer" and "Ultra View Universal DAB Detection Kit" (Ventana) were used. Counterstaining was performed with Mayer's hematoxylin solution. For quantification, the numbers of APP-positive axonal swellings were counted in matching sections.

Mixed cell culture

Cells were prepared from 1 to 2 day old mice. After decapitation brains were transferred into HBSS containing 10 mM HEPES. The meninges were removed and brains were minced, washed and incubated for 25 min in HBSS/10 mM HEPES with 0.5 mg/ml papain (Sigma Aldrich) and 10 μg/ml DNAse (Roche Diagnostics, Mannheim, Germany). Cells were washed again in BME (Life Technologies) media, dissociated and then plated at a density of 3×10^5 cells/ml and cultured in BME media supplemented with 10% FCS and penicillin/streptomycin.

Neuronal cell culture

Cortical tissue was obtained from C57BL/6J mice at embryonic Theiler stage 24 (gestational day 16). After removal of the meninges the tissue was digested with 1 mg/ml trypsin followed by dissociation. After washing cells were seeded in Neurobasal media (Gibco) supplemented with B27 (Invitrogen), L-glutamine (Invitrogen), HEPES (Invitrogen) and gentamycin sulfate.

Glutamate excitotoxicity

Seven day old neuronal cultures were washed with PBS of 37 °C. Next neurons were incubated with DMEM (Gibco) in the absence or presence of 1 μM, 10 μM or 100 μM glutamate for 4 h at 37 °C. Supernatants were collected and cell death was measured by LDH-Cytotoxicity Kit (Roche Diagnostics).

Immunofluorescence staining

Neuronal cultures grown on coverslips were washed with PBS, fixed with 4% PFA and permeabilized with 0.2% Triton-X 100. For histologic analysis of mouse brains, animals were perfused with 4% buffered formalin. Immunofluorescence staining was performed by incubation with mouse antibody directed against NSE (Dakoytomation, Glostrup, Denmark; 1:100) and chicken antibody directed against MAP2