

Aldolase C/Zebirin II is Released to the Extracellular Space after Stroke and Inhibits the Network Activity of Cortical Neurons

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Abstract Cell death after stroke involves apoptotic, autophagocytic and necrotic mechanisms which may cause the release of cytosolic proteins to the extracellular space. Aldolase C (AldC) is the brain specific isoform of the glycolytic enzyme fructose-1,6-bisphosphate aldolase. According to its characteristic striped expression pattern in the adult cerebellum AldC is also termed zebirin II. Here, we demonstrate release of AldC into the cerebrospinal fluid (CSF) after stroke in

vivo. Studies with cell cultures confirmed that AldC is released to the extracellular space after hypoxia. Moreover, addition of purified recombinant AldC to networks of cortical neurons plated on multielectrode arrays reversibly inhibited the spontaneous generation of action potentials at AldC concentrations which can be expected to occur after lesions of the human cerebral cortex. This mechanism could be relevant in the pathogenesis of the electrophysiological changes in the penumbra region after stroke.

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Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
A.d.	Aqua dest (H ₂ O)
AldA	Aldolase A
AldB	Aldolase B
AldC	Aldolase C
BSA	Bovine serum albumin
CSF	Cerebrospinal fluid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
FBP	Fructose-1,6-bisphosphate
GAP	Glycerinaldehyde-3-phosphate
GDH	Glycerol-3-phosphate-dehydrogenase
IPTG	Isopropyl-β-D-thiogalactopyranosid
kDa	kilo Dalton
LB	Luria Bertani
MRI	Magnetic resonance imaging
PBS	Phosphate buffered saline
PMSF	Phenyl methyl sulfonyl fluoride
RT	Room temperature

SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TIM	Triose-phosphate-isomerase
TTX	Tetrodotoxin
WB	Western blot

Introduction

Cell death after stroke is a multifaceted process. It is thought to involve apoptotic, autophagocytic and necrotic mechanisms. Furthermore, functional damage of neurons commonly occurs after stroke as evidenced from studies in animal models of stroke and from functional imaging data in human patients. These show that the necrotic core of ischemic brain lesions is surrounded by a zone of compromised tissue which is called penumbra [1] defined as electrically unexcitable but still viable cells which may be rescued from infarction by appropriate therapy [2, 3]. Furthermore, this area is characterized by an intermediate decrease of cerebral blood flow leading to transient episodes of relative tissue hypoxia [1]. Intense efforts are undertaken currently to understand the molecular basis of cell death since therapeutic interventions interfering with these mechanisms would be of considerable clinical benefit.

Aldolase is an enzyme of the glycolytic pathway which catalyzes the aldol cleavage of fructose-1,6-bisphosphate (FBP) into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate. In mammals there exist three isoforms of the enzyme termed Aldolase A, B and C (abbreviated subsequently AldA, AldB, and AldC). AldA is found mainly in skeletal muscle and to a lower extent in brain, AldB is present in the liver [4, 5] and AldC is the brain-specific isoform [6]. The crystal structures of AldA from rabbit muscle and AldB from human liver [7] revealed tetrameric structures of these enzymes. In addition to homo-tetramers, AldA–AldC hybrids have been found in brain tissue using isoelectric focussing (compare [8] and references therein). In the developing rodent forebrain, AldC mRNA is expressed strongly in the subventricular zone in newborn animals [9]. The transcript appears in Purkinje cells of the cerebellar vermis at postnatal day 5 (P5) and becomes more widely expressed at P12 [10]. After postnatal day 15 the expression in the cerebellum becomes restricted to a subpopulation of Purkinje cells [10], where it is distributed in alternate positive and negative stripes. Interestingly, this zebra-like staining pattern was initially found using a monoclonal antibody which was generated

against a crude homogenate of cerebellum and the antigen recognized by this antibody was accordingly termed zebrin II [11]. So far, the functional relevance of this striking expression pattern in the cerebellum is not known. Expression of AldC is also found in the cerebral cortex, striatum, hippocampus, hypothalamic nuclei, primary olfactory cortex (at lower levels) [12] and the spinal cord. In the forebrain, astrocytes show an immunocytochemical staining of AldC [9, 13, 14] and high concentrations of AldC were measured in the human cerebral cortex (6520 ng AldC/mg protein) [15].

Release of AldC to the extracellular space of the central nervous system has been found to take place in a variety of disease states of the brain. In patients with severe head injury a slight rise of AldC in blood and cerebrospinal fluid (CSF) was reported that reached its peak on the third day after the lesion, and became normal around the eighth day [16]. On the other hand, AldC was found to be expressed in different types of cerebral tumors including astrocytomas, glioblastomas and medulloblastomas [17, 18]. Taken together, these findings clearly indicate that AldC can be released from the cytosol to the extracellular space under certain conditions. Therefore, AldC was discussed as a marker for brain damage in different lesion types.

In the present study, we examine the possible role of AldC release to the extracellular space in stroke.

Materials and methods

Patients and cerebrospinal fluid samples

Cerebrospinal fluid was obtained by atraumatic lumbar puncture from three different individuals suffering from acute stroke and from probands without pathology of the central nervous system based on their informed consent. The amount of CSF obtained routinely was 2–3 ml divided in two fractions, one of which was used for cytological analysis and the other was used for biochemical studies (protein concentration, IgG index, oligoclonal bands). Our analyses were done with the latter fraction.

Patient 1 was a female aged 25 years suffering from a right-sided hemiplegia and aphasia caused by a partial infarction of the left medial cerebral artery as shown by MRT and CT. CSF analysis 13 days after the onset revealed 17 cells/ μ l and protein concentration was found to be 50 mg/dl.

Patient 2 was a 39 year old male who presented with a mild left-sided hemiparesis which was caused by an infarction of the right medial cerebral artery according to MRT. Angiography revealed Moya-Moya

syndrome. In the CSF, cell count and protein concentration were normal 6 weeks after the onset.

Patient 3 was a female aged 50 years with an acute right-sided hemiplegia and global aphasia based on a combined infarction of left anterior cerebral artery and the left medial cerebral artery as evidenced by MRT and MR angiography. Examination of the CSF 5 days after the onset revealed normal cell counts and normal total protein.

Cloning of an AldC expression plasmid

The cDNA of rat AldC was amplified by polymerase chain reaction with *Taq*-Polymerase (Fermentas, St. Leon-Rot; 35 PCR cycles: 95°C for 3 min, 94°C for 1 min, 55°C for 2 min, 70°C for 4 min and 72°C for 5 min) with the primer sequences rAldC-5'-BamHI (5'-GGAGGATCCATGCCCACTCATAAC-3') and rAldC-3'-SacI (5'-CTGGAGCTCTCAGTAGGCA TGGTTGG-3') using a cDNA mixture prepared from total RNA of rat brain [19] by oligo-dT-primed reverse transcription with SuperscriptII (Invitrogen) as template. The purified PCR fragment was ligated into the pQE80L expression Vector (Qiagen) and sequenced (ABI Prism 310 Genetic Analyzer; Perkin Elmer, Weiterstadt, GER). The following oligonucleotides were used in this study: pQE80L-F (5'-CGGATAACAATTC ACACAG-3'), pQE80L-Seq-AS2 (5'-CAACGGTGGTATATCCAG-3'), S-RNALDCR-BamHI (5'-GGAGGATCCATGCCCACTCATAAC-3'), AS-RNALDCR-SacI (5'-CTGGAGCTCTCAGTAGGATGGTTGG-3'), AldC-Seq-S1 (5'-GTGCCTCTAGCTGGGAC-3'), AldC-Seq-S2 (5'-CATCTCTCCACGAGAC-3'), AldC-Seq-AS3 (5'-GGGCACAGTTCGACGCAG-3'), AldC-Seq-S4 (5'-GCACTCAGTGCCTGGAGAGG-3'), AldC-Seq-AS5 (5'-CGGCGATTCTCCTCAGTGTTC-3').

Bacterial expression and purification of recombinant proteins

AldC was expressed as an N-terminally HIS₆ tagged protein in *Escherichia coli* BL21 and purified as described [20].

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic separation of proteins followed the standard method according to [21] by means of denaturing discontinuous 10% SDS-polyacrylamide gel electrophoresis. The gels were either stained with

Coomassie blue or further examined by Western blotting (WB).

Western Blotting

After the electrophoretic separation by SDS-PAGE (10%) proteins were blotted on a nitrocellulose membrane (Schleicher&Schuell) for 1 h at 0.8 mA/cm² using a semidry blotter (Biorad), stained with Ponceau S and blocked with 3% BSA in TBS/Tween 20 (0.05% v/210 v) at 4°C over night. The first antibody (mouse monoclonal anti-poly-Histidine; 1:2000; Sigma) was added to the membrane and incubated for 2 h at room temperature (RT). After washing the membrane in TBS/Tween20 (0.05% v/v) the secondary antibody (anti-mouse-peroxidase-coupled IgM & IgG, 1:10,000; Dianova) was incubated for 1 h at RT. After washing four times with TBS/Tween20 (0.05% v/v) the bound antibodies were visualized using enhanced chemiluminescence (ECL) [22]. The exposure time varied between 5 s and 2 min. WB-Analysis of the CSF for AldC was performed with the monoclonal Zebrin II antibody (1:4000; incubation time 2 h at RT) and anti-mouse-peroxidase-coupled IgM & IgG in ratio of 1:10,000 as second antibody. Proteins from CSF were precipitated with acetone prior to SDS-PAGE [23].

Kinetic aldolase activity assays

The activity of AldC was determined at 37°C in a coupled kinetic assay with glycerol-3-phosphate dehydrogenase (GDH) and triose-phosphate-isomerase (TIM). The assay was performed in a 96 microtiter plate (Sarstedt) in 100 mM Tris/HCl pH 7.3, 0.4 mM NADH/H⁺ (Boehringer Ingelheim), 4 IU/ml GDH (Roche), 10 IU/ml TIM from rabbit muscle (Sigma) and 5 mM FBP (Sigma) in a total volume of 200 µl. The amount of added recombinant AldC ranged from 0.25 µg to 2 µg per 200 µl test volume. The reactions were started by addition of FBP and the time course of the absorbance at 340 nm was measured in intervals of 20 s (15 or 20 measurements) using a microplate reader (Benchmark, Biorad). The velocity of NADH consumption was determined using linear curve fitting (Excel, Microsoft). All measurements were performed in triplicate.

Electrophysiology

Electrophysiological measurements using multielectrode arrays (MCS, Reutlingen/Germany) and cultures of cryopreserved cortical neurons of the embryonic rat

(QBM, Ontario, Canada) were performed as described previously [24]. The rate of spontaneous action potentials, burst activity and interburst time intervals were calculated using the software SpANER (Result GmbH/Tönnisvorst/Germany). Test substances were applied directly into the recording medium (Temp. 37°C) in the final concentrations as indicated. After each run the neurochip was rinsed with bath solution to control the functional recovery.

Induction of cell death in neural cells

B35 neuroblastoma cells were grown in DMEM/F12 (Invitrogen) supplemented with 5% fetal calf serum, 0.3 g/l L-glutamin, 100 U/ml Penicillin and 100 µg/ml Streptomycin. The cells were plated in 6-well plates at a density of 1×10^6 cells per well. After 14 h the cells were washed with PBS and hypoxia was induced by addition of KCN (0, 0.5, 1 and 2 mM) in DMEM/F12 and programmed cell death was triggered with C2-ceramide (Sigma; 0, 15 and 30 µM final concentration in DMEM/F12 added from a stock-solution in ethanol). After 24 h tissue supernatants were centrifuged at 1,500g for 15 min at 4°C and the supernatants were analyzed for aldolase activity.

Results and discussion

Detection of AldC in the cerebrospinal fluid of three patients after stroke

To screen for conditions which cause the release of AldC to the extracellular space *in vivo* we first examined a panel of CSF samples from 7 probands without pathology of the central nervous system using WB with the Zebrin II antibody. However, in none of these samples AldC immunoreactivity was detectable (Fig. 1A) indicating that under normal conditions only low amounts AldC are present in the adult CSF. This contrasted with the presence of AldC in 3 CSF samples of patients suffering from stroke. The highest amount of AldC was found in CSF which was collected 5 days after a severe infarction in the territory of left anterior cerebral and medial cerebral artery (patient 3, Fig. 1A). A somewhat lower amount of AldC was detected in the CSF of Patient 1 who suffered from an extended lesion of the left medial cerebral artery and in whom CSF was collected 13 days after stroke. The lowest amount of AldC was detected in the CSF sample of patient 2 who was affected with a partial infarction of the right medial cerebral artery and where CSF was collected 6 weeks after disease onset. Thus, in

general the amount of AldC in the CSF corresponded to the severity of the brain lesions. Interestingly, even six weeks after disease onset AldC was detectable in the CSF of patient 2. This qualitative correlation suggests that AldC in the CSF may be a useful marker for cell damage in stroke.

Release of aldolase in cell cultures

In agreement with the clinical data described above *in vitro* experiments using B35 neuroblastoma cells as a culture model confirmed that AldC can be released to the extracellular space after hypoxia in order to trigger necrosis. These cells express AldC and when they were subjected to acute chemical hypoxia by adding cyanide to the culture medium, the aldolase activity released to the extracellular compartment was increased up to 6-fold (Fig. 1B). Interestingly, aldolase activity was detectable in cell culture supernatants of B35 neuroblastoma cells which were maintained in serum-free medium, even when the cells were not exposed to KCN (Fig. 1B). Surprisingly, after application of 0.5 mM KCN higher amounts of aldolase activity were

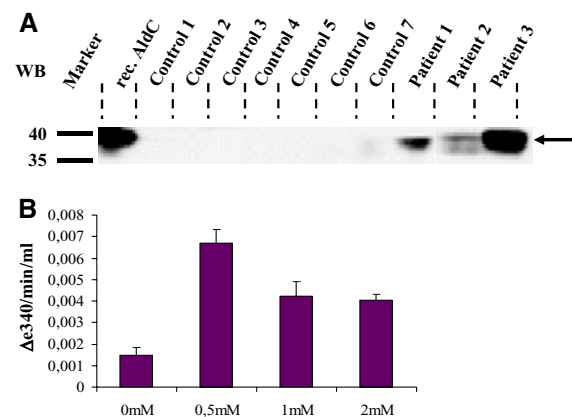


Fig. 1 Release of aldolase to the extracellular space after cell death. **(A)** Western Blot analysis of aldolase C in cerebrospinal fluid. Cerebrospinal fluid samples of 10 subjects were analyzed for AldC by Western-blotting using the zebrin II antibody after precipitation with acetone and separation by SDS-PAGE (10%). Sample numbers are indicated at the top of the lanes. Control 1 to control 7 refer to cerebrospinal fluid samples of probands with no pathology of the CNS, patient 1 to patient 3 refer to specimen of stroke patients. Bars on the left indicate the position of apparent molecular weight marker bands (in kDa). The arrow on the right side (→) indicates the position of the AldC. The positive control was recombinant AldC. **(B)** Aldolase activity in the supernatant after treatment of B35 neuroblastoma cells with KCN. B35 cells were treated with 0 mM, 0.5 mM, 1 mM and 2 mM KCN. After cell-death the supernatant was analyzed for aldolase activity. Aldolase-activity was plotted against KCN-concentrations. All determinations were performed in triplicate (bars, mean; error bars, SD)

detectable in the supernatants than at higher concentrations. To some extent this may be the consequence of an inhibition of the activity of AldC at higher concentrations of KCN (compare Supplemental Material 3). On the other hand, at lower concentrations of KCN the cells may have time to react to the hypoxic condition prior to cell death. Since it is known that hypoxia can induce the expression of AldC [25] this may lead to enhanced release of aldolase in the presence of 0.5 mM KCN. Interestingly, when programmed cell death was induced after addition of ceramide to the cultures, the amount of AldC in the supernatant was increased only marginally (data not shown).

Influence of extracellular aldolase C on the spontaneous electrophysiological activity of cortical neurons

To screen for functional effects of AldC in the extracellular space the recombinant enzyme was purified after expression in BL21 cells by affinity chromatography (Supplemental material 1A, B, C). The purified enzyme was active (Supplemental material 1D, E) and formed tetramers according to gel

filtration chromatography (Supplemental material 2). Cultures of cortical neurons were exposed to increasing concentrations of recombinant AldC (100 nM–10 μ M) in the culture medium. These neuronal networks develop spontaneous rhythmic discharges which were recorded by an extracellular electrode array [24]. After addition of AldC to the culture supernatant the rate of spontaneous action potentials decreased significantly in a reversible manner (Fig. 2A). AldC (1 μ M) caused a clear inhibition of the rate of spontaneous action potentials. After incubation with 10 μ M AldC for 20 min the generation of action potentials was nearly abolished. When the recombinant protein was washed out subsequently, the formation of action potentials resumed. As a control, BL21 cells which did not express AldC were lysed, proteins were chromatographed on a HiTrap chelating column and eluate fractions were dialyzed. Incubation of neuron cultures with this control preparation from BL21 cells which did not express recombinant AldC had no significant effect on action potential generation (Fig. 2B). When the relative change of the spike rate was plotted against the concentration of AldC a clear dose-response

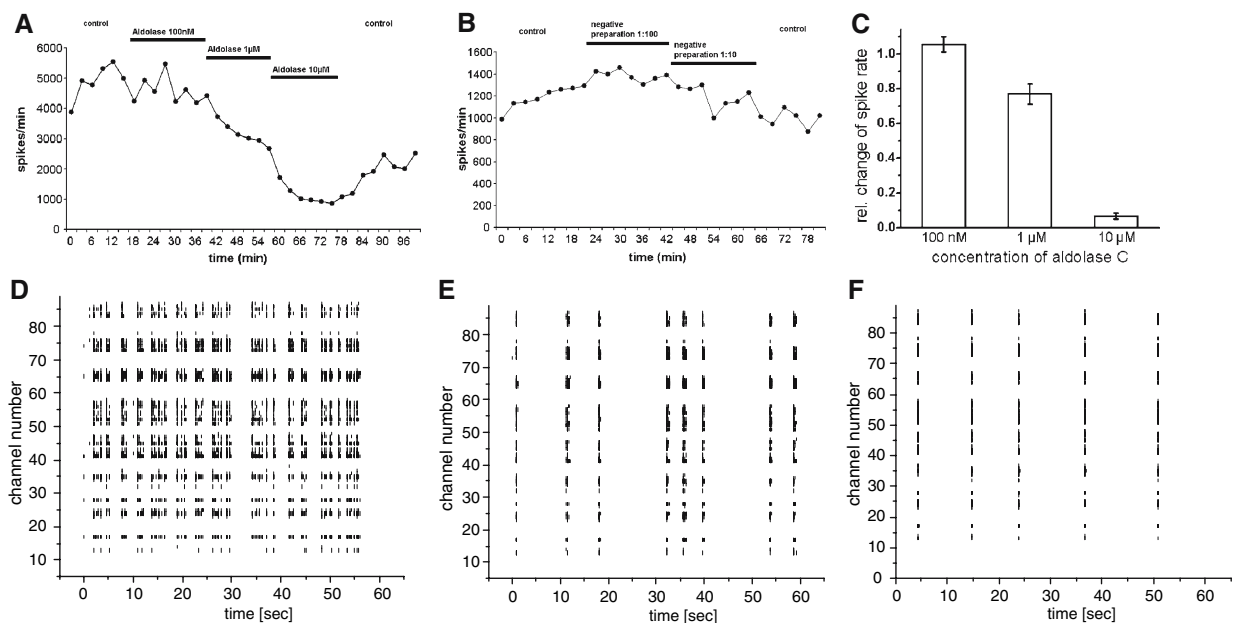


Fig. 2 Influence of extracellular aldolase C on the generation of action potentials by cortical neurons. **(A)** Different concentrations of recombinant AldC obtained after purification with a Ni-HiTrap column and dialysis were added to cultures of cortical neurons grown on an electrode array chip at the concentrations and for the time intervals indicated at the black horizontal bars. **(B)** Control: as a negative control eluates from a Ni-chelating HiTrap were prepared from BL21 bacteria which did not express AldC and dialyzed. The volumes added corresponded to

the volumes of the recombinant AldC solution used in panel A. **(C)** Dose-response relationship for the relative change of the spike rate for different concentrations of AldC as indicated below. Bars represent the mean, error bars represent the standard deviation ($n = 28$). **(D–F)** Spike raster plots from cortical neurons before **(D)** and after application of 1 μ M **(E)** and 10 μ M AldC **(F)** revealed the dose-dependent global reduction of spontaneous spike activity (each bar represents a spike)

relationship was apparent (Fig. 2C) which was also evident in spike raster plots (Fig 2D, E, F).

Functional relevance of aldolase C in stroke

The specific effect of AldC on the spontaneous activity of neuronal cortical networks provides evidence for a strong functional side effect of this protein after binding to the cell surface (Fig. 2). The sensitivity of neuronal networks on multielectrode arrays to detect neuroactive substances is very high and the setup was recommended to be used as a biosensor [26]. E.g. TTX the specific sodium channel blocker could be detected in nM concentrations on the chip [24]. Here, different mechanisms of the suppressing effect are conceivable. AldC may act on voltage-gated or receptor operated ion channels or by modulation of the synaptic efficacy. The elucidation of the molecular mechanism of this effect, however, was beyond the scope of this report and studies in the future will be needed to establish the target of extracellular AldC. The observation that the extracellular application of AldC to functional networks of cortical neurons reversibly suppressed the formation of action potentials (Fig. 2) indicates a possible significance of AldC release in the pathophysiology of stroke. Based on the data of Haimoto and Kato [15] one can calculate that the concentration of AldC in the human cerebral cortex is approximately 1.17 mg per g wet weight. This would correspond to a concentration of ca. 30 μ M AldC, a value which is three times higher than the concentration of AldC we found to nearly abolish the generation of action potentials in cortical neurons (Fig. 2). This concentration may be even higher under hypoxic conditions (see above, [25]). Since the extracellular space in adult brain is a small compartment estimated to occupy a fraction of approximately 20% of the total brain volume [27] local release of AldC in a concentration of 30 μ M or higher from a focus of necrotic cells is likely to propagate relevant extracellular concentrations of AldC around the lesion. This is consistent with the detection of AldC at rather distant sites after brain lesions as in the CSF after lumbar puncture. On the other hand, interactions of AldC with glycosaminoglycans of the extracellular matrix [28] could retain relevant amounts of AldC near the sites of release. It is known that the necrotic core of ischemic lesions is surrounded by a region characterized by reversible functional alterations, the penumbra, and that reductions of cerebral blood flow correlate with these changes [1]. Our data suggest that hemodynamic mechanisms are not the only cause of functional changes in the penumbra. As an additional mechanism

the necrotic core of an ischemic brain lesion can release molecules which may contribute to electrophysiologic changes in the penumbra. In the light of this novel mechanism of AldC in stroke a broader prospective study of AldC release to the CSF in patients after ischemic lesions, immunocytochemical demonstration of AldC in experimental stroke lesions in an animal model and studies probing the molecular targets of AldC seem desirable in the future.

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