

Kinetics and activity of arylsulfatase A in leukocytes derived from patients with cerebral palsy

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Activity and kinetics of arylsulfatase A (ASA, EC 3.1.6.8) were analyzed in leukocyte homogenates derived from patients suffering from cerebral palsy. Lower ASA activity was found in the patients' leukocytes than in controls, as determined by spectrophotometry using chromogenic substrate *p*-nitrocatechol sulfate (*p*-NCS). Kinetic parameters, K_m and v_{max} , for leukocyte ASA were determined from the dependence of initial reaction velocities on the *p*-NCS concentrations. A slight difference in K_m values was found for leukocyte enzyme in cerebral palsy (0.26 mmol L⁻¹) compared to the control (0.21 mmol L⁻¹), whereas v_{max} value for leukocyte ASA in disease reached only 58% of the control value. In addition, the presence of the most common mutations associated with ASA pseudodeficiency (N350S, 1524+95 A>G) and metachromatic leukodystrophy (P426L) was detected in all investigated patients. Changes in activity and kinetic parameters of leukocyte ASA in cerebral palsy are most probably related to the decrease of enzyme concentration; the detected mutations might at least partially contribute to the observed changes.

Keywords: arylsulfatase A, kinetic parameters, enzyme activity, mutations in ASA gene, cerebral palsy

Lysosomal arylsulfatase A (ASA, EC 3.1.6.8) is a member of a large sulfatase family – enzymes hydrolyzing sulfate ester bonds in numerous structurally very different sulfated compounds (1). Crystal structure of human arylsulfatase A was determined in 1998 and the catalytic mechanism of ASA was suggested and described in detail (2). Physical and chemical properties of ASA have been extensively studied in biological samples of different species (1–4). Also, the catalytic mechanism of ASA was confirmed by kinetic studies of numerous active site mutants (5, 6). However, only a few reports dealing with ASA kinetics in human disorders with decreased ASA activity have been published (7–10).

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Activity and kinetics of ASA are studied using synthetic substrates: chromogenic *p*-nitrocatechol sulfate or fluorogenic 4-methylumbelliferyl sulfate. The major physiological substrate of lysosomal ASA is cerebroside-3-sulfate, an important lipid constituent of oligodendrocyte membranes contributing to the maintenance of myelin sheath integrity. ASA deficiency causes metachromatic leukodystrophy (MLD), a rare autosomal recessive disorder characterized by the storage of cerebroside sulfate mainly in the nervous tissue. Pathologically, a progressive demyelination occurs, causing various neurological symptoms such as pyramidal and extrapyramidal signs, progressive spastic quadriplegia, *etc.* (1).

In addition to mutations found to be the most frequent ones in European MLD patients (P426L and 459+1G>A) and mutations responsible for the majority of arylsulfatase A pseudodeficiency (ASA-PD) alleles (N350S and 1524+95 A>G), a large number of other mutations and polymorphisms in the ASA gene, resulting in different levels of decreased enzyme activity, have been reported so far (11–13). However, only several mutations have been characterized with regard to catalytic properties of the enzyme due to changes in either conformation or concentration of the enzyme (14).

Cerebral palsy is a neurodevelopmental disorder with complex etiopathogenesis. It affects body movement and muscle coordination and is caused by the damage and/or faulty development of motor areas in the brain. Previous clinical observations have showed that arylsulfatase A activity is decreased in leukocytes derived from patients suffering from cerebral palsy (15). According to available data, arylsulfatase A kinetics has not yet been studied in human leukocytes derived either from patients with cerebral palsy or other disorders accompanied by decreased ASA activity. The aim of this preliminary study was to determine the kinetic parameters of leukocyte ASA and to elucidate whether possible changes of ASA kinetic properties may explain the decreased leukocyte ASA activity in patients with cerebral palsy.

EXPERIMENTAL

Materials

Substrate for determination of the enzyme activity and the kinetic assay, *p*-nitrocatechol sulfate (*p*-NCS), was purchased from Sigma-Aldrich (USA). Bovine serum albumin (BSA) of 98% purity was also obtained from Sigma-Aldrich. Specific primers, DNA standards and restriction enzymes were purchased from Invitrogen and Promega (USA). The PCR amplification mixture was purchased from Takara Bio. Inc. (Japan). All other chemicals were of analytical grade.

Subjects

Blood samples were obtained by venipuncture from individuals with cerebral palsy ($N = 4$; age range: 3–10 years; 2 females, 2 males) and healthy individuals ($N = 3$; age range: 10–26 years; 2 females, 1 male). Blood was collected in duplicate during morning hours and before giving any medications. The local ethical committee approved the study.

Isolation of leukocytes

A slightly modified method of Haltia *et al.* and Bass *et al.* was used for isolation and homogenization of leukocytes (16, 17). Briefly, 5–7 mL of venous blood was drawn to a test tube containing 1 mL of dextran. The samples were allowed to stand at room temperature for 45 minutes for sedimentation of erythrocytes and the suspension of leukocytes. Pure leukocyte pellets were obtained after centrifugation of the upper plasma layer, separation of leukocyte pellets from the serum, and additional washing with haemolytic buffer. Leukocyte pellets were dispersed in 0.25% Triton X-100 and homogenized. Determination of the protein content in leukocyte homogenates was performed according to Lowry's method using bovine serum albumin (BSA, 1 mg mL⁻¹) as a standard (18).

Determination of ASA activity

The activity of arylsulfatase A was determined in leukocyte homogenates by spectrophotometry ($\lambda = 515$ nm) using 10 mmol L⁻¹ *p*-nitrocatechol sulfate (*p*-NCS) as a chromogenic substrate, as described by Jordan *et al.* (19). The ASA activities were expressed as nanomoles of the formed *p*-nitrocatechol (*p*-NC) per mg protein per hour.

ASA kinetic analysis

Assays were conducted at 36.5 °C in a final volume of 700 μ L, containing aliquots of leukocyte homogenate (75 μ g of protein) and substrate (*p*-NCS) solution. The substrate was dissolved in 0.5 mol L⁻¹ acetate buffer (pH 5.0) containing 0.5 mmol L⁻¹ sodium pyrophosphate (Na₄P₂O₇) and 10% sodium chloride. The substrate concentration ranged from 0.2 to 9.0 mmol L⁻¹. Absorbance of the released *p*-NC was continuously monitored for 5 min at 515 nm and the reaction rate was determined from the initial slope. Absorption measurements were performed against appropriate substrate blanks on a Unicam UV/Vis spectrophotometer UV4-100 (UK) with thermostated cell holders in 1 cm silica-glass cells. Measurements were performed in duplicate for each substrate concentration. Kinetic parameters, K_m and v_{max} , were calculated by the non-linear regression analysis from the dependence of the initial reaction rate on the substrate concentration. This calculation was performed using the PeakFit software, which – by the iteration procedure – calculates the most correct v_{max} and K_m values based on experimental points and the Michaelis-Menten equation. Calculated v_{max} values were expressed in mmol L⁻¹ of *p*-NC liberated per minute at 36.5 °C per mg of total leukocyte proteins.

DNA isolation and PCR amplification

Genomic DNA was extracted from leukocytes and fragments of the ASA gene were amplified using specific primers (11, 12).

Determination of the N350S and 1524+95 A>G mutations associated with arylsulfatase A pseudodeficiency

The N350S and 1524+95 A>G mutations were determined according to the previously described procedure (12). Briefly, PCR products were digested with *BsrSI* and *DdeI*, respectively. In the presence of the N350S mutation, the 275 bp fragment A (allele 2) is cleaved to two smaller fragments of 161 bp and 114 bp (allele 1). Fragment B of 114 bp (allele 2) generates the 97 bp fragment (allele 1) in the presence of the 1524+95 A>G mutation.

Determination of the P426L mutation associated with metachromatic leukodystrophy

The P426L mutation was analyzed using the restriction enzyme *PstI*, as previously described (11). In the presence of the mutation, the 100 bp fragment (allele 2) is cleaved to 70 bp and 26 bp (allele 1).

Statistics

Student's *t*-test (Cochran and Cox modification) was used.

RESULTS AND DISCUSSION

Kinetic parameters of arylsulfatase A in leukocytes

In our study of leukocyte ASA kinetics, the initial reaction rate was found to be lower in leukocytes of the patients with cerebral palsy than in the controls, as shown by the progress curves of the ASA catalyzed reactions (Fig. 1). Results of the performed ASA kinetic study showed that, in case of leukocytes of both healthy individuals and patients, K_m values of the enzyme toward *p*-NCS are in the range previously reported for ASA in human tissues (0.20–0.60 mmol L⁻¹) (Table I) (20). Also, comparable values for the K_m of ASA from patients' leukocytes ($K_m = 0.26$ mmol L⁻¹) and control leukocytes ($K_m = 0.21$ mmol L⁻¹) might indicate that no change in enzyme affinity of ASA occurred in spastic cerebral palsy (Table I). On the other hand, a significant decrease of the v_{max} value was found in cerebral palsy (8.44 mmol L⁻¹ min⁻¹ mg⁻¹) compared to the control value (14.53 mmol L⁻¹ min⁻¹ mg⁻¹) (Fig. 2 and Table I).

Table I. Kinetic parameters of arylsulfatase A in leukocytes derived from healthy individuals and patients with the diagnosis of cerebral palsy

	K_m (mmol L ⁻¹) ^a	v_{max} (mmol L ⁻¹ min ⁻¹ per mg protein) ^a
Control (<i>N</i> = 3)	0.21 ± 0.02	14.53 ± 2.02
Cerebral palsy (<i>N</i> = 4)	0.26 ± 0.03	8.44 ± 0.76 ^b

N – number of individuals.

^a Mean ± SD.

^b Statistically significant difference in comparison with the control (*p* < 0.01).

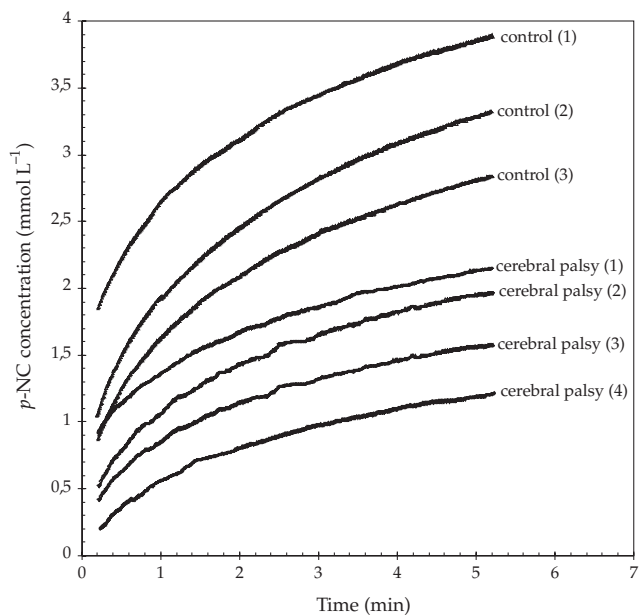


Fig. 1. Progress curves of the ASA catalyzed reactions showing individual differences between samples derived from the controls and patients with cerebral palsy ($c(p\text{-NCS}) = 7.01 \text{ mmol L}^{-1}$, $36.5 \text{ }^\circ\text{C}$, $\text{pH } 5.0$).

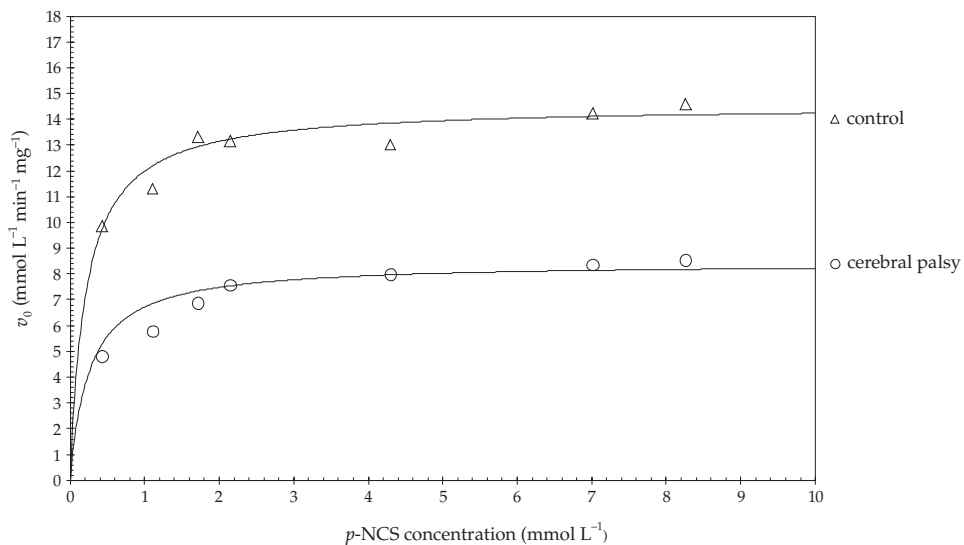


Fig. 2. Dependence of the initial reaction rate on the substrate concentration ($36.5 \text{ }^\circ\text{C}$, $\text{pH } 5.0$, duplicate measurements). v_{max} values are expressed as mmol L^{-1} of $p\text{-NC}$ liberated per minute per mg of total leukocyte proteins.

Only a few studies have dealt with the kinetics of arylsulfatase A in human disorders characterized by low ASA activity and caused by mutations in the ASA gene (7–9). In those studies no substantial changes in K_m , pH optima or heat stability of the enzyme analyzed from skin fibroblasts were found between the controls and patients with ASA pseudodeficiency or metachromatic leukodystrophy. However, a more recent study, also performed in skin fibroblasts derived from individuals with ASA pseudodeficiency and controls, showed significant kinetic differences in both K_m and heat inactivation properties of the enzyme in ASA pseudodeficiency (10). In our preliminary study of ASA kinetics in leukocytes, the reaction of ASA with *p*-nitrocatechol sulfate was followed directly from leukocyte homogenates derived from patients with cerebral palsy and from healthy individuals. Experimental conditions were similar to those routinely used for determination of leukocyte ASA activity for diagnostic purposes. In such conditions, optimal for the ASA activity, the activity of another lysosomal hydrolase, arylsulfatase B (ASB), is minimized due to lower substrate concentration, lower pH and the presence of sodium pyrophosphate and sodium chloride in the reaction mixture (20–22).

The presence of the N350S, 1524+95 A>G, P426L mutations and arylsulfatase A activity

The observed decrease of the leukocyte ASA v_{max} in spastic cerebral palsy may be related to the confirmed presence of three different mutations in the ASA gene. Namely, additional genotyping was performed for the most common mutations associated with arylsulfatase A pseudodeficiency (N350S and 1524+95 A>G mutations), and metachromatic leukodystrophy (P426L mutation). The results showed that each patient with the diagnosis of spastic cerebral palsy was a heterozygous carrier of all three analyzed mutations, while healthy individuals were found to be homozygous for normal alleles (Fig. 3 and Table II). Heterozygosity for a single analyzed mutation does not necessarily result in decreased enzyme activity. However, there is high probability that heterozygosity for three different mutations in the arylsulfatase A gene would have an impact on the ASA activity. This was indeed confirmed by a spectrophotometric determination of ASA activity in leukocytes, showing that in samples derived from patients with cerebral palsy arylsulfatase A decreased significantly and reached only 31% of the activity measured in control samples (Table III).

Table II. Arylsulfatase A pseudodeficiency and metachromatic leukodystrophy genotypes in patients with the diagnosis of cerebral palsy and in healthy individuals

	Genotype ^a								
	N350S			1524+95 A → G			P426L		
	1,1	1,2	2,2	1,1	1,2	2,2	1,1	1,2	2,2
Cerebral palsy (N = 4)	0	4	0	0	4	0	0	4	0
Control (N = 3)	0	0	3	0	0	3	0	0	3

N – number of individuals.

^a 1,1 – homozygous for mutant sequence; 1,2 – heterozygous; 2,2 – homozygous for normal sequence.

Table III. Arylsulfatase A activity in leukocytes derived from healthy individuals and patients with the diagnosis of cerebral palsy

	ASA activity (nmol h ⁻¹ mg ⁻¹) ^{a,b}
Control (N = 3)	125 ± 27
Cerebral palsy (N = 4)	39 ± 5 ^c

N – number of individuals.

^a The ASA activity is expressed as nanomoles of the formed *p*-nitrocatechol (*p*-NC) per mg protein per hour.

^b Mean ± SD.

^c Statistically significant difference in comparison with the control ($p < 0.05$).

The decrease in ASA activity, as well as the decrease in the v_{\max} value for leukocyte ASA in cerebral palsy, most probably reflect a decrease of the total enzyme concentration. However, a change in the enzyme conformation as a consequence of the present mutations could not be completely excluded. Namely, changes of the enzyme biochemi-

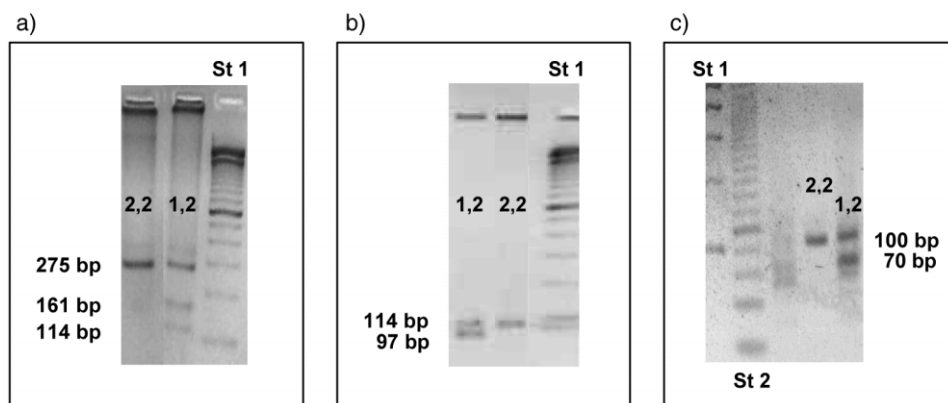


Fig. 3. Specific restriction products shown in several analyzed samples derived from the controls and subjects with the diagnosis of cerebral palsy: a) detection of the N350S mutation; b) detection of the 1524+95 mutation A>G; c) detection of the P426L mutation. St 1 – DNA standard (100 bp); St 2 – DNA standard (25 bp); 1,2 – heterozygous; 2,2 – homozygous for normal sequence.

cal function resulting from the here analyzed mutations have been well described, causing either decreased stability of the enzyme within lysosomes (P426L mutation), or loss of the polyadenylation site leading to reduction of ASA mRNA (1524+95 A>G mutation) and loss of the *N*-glycosylation site (N350S mutation), with consequent probable disturbed targeting of the protein at lysosomes (23–25).

CONCLUSIONS

Decreased activity and v_{\max} of leukocyte ASA in cerebral palsy are most probably related to the decrease of enzyme concentration in disease, while the detected mutations may contribute to the observed changes. Future research is directed to optimization of the method used for the analysis of arylsulfatase A kinetic parameters directly from human leukocytes, which are easily and less aggressively obtained for diagnostic procedures compared to bioptic material (skin fibroblasts). Such an optimized method for the analysis of leukocyte ASA kinetics may provide additional information and a better insight into the mechanism of the altered activity of this enzyme occurring in cerebral palsy.

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S A Ž E T A K

Kinetika i aktivnost leukocitne arilsulfataze A u osoba s dijagnozom cerebralne paralize

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Analizirane su aktivnost i kinetika arilsulfataze A (ASA, EC 3.1.6.8) u leukocitnim homogenatima osoba oboljelih od cerebralne paralize. Spektrofotometrijskim određivanjem aktivnosti ASA prema kromogenom supstratu *p*-nitrokatehol sulfatu (*p*-NCS) utvrđene su smanjene aktivnosti enzima u leukocitima oboljelih osoba. Kinetički parametri, K_m i v_{max} , leukocitne ASA određeni su iz ovisnosti početne brzine reakcije o koncentraciji *p*-NCS. Utvrđena je manja razlika između K_m vrijednosti enzima zdravih (0.21 mmol

L^{-1}) i oboljelih osoba (0.26 mmol L^{-1}), dok je vrijednost v_{\max} enzima u oboljelih iznosila 58% vrijednosti v_{\max} enzima zdravih osoba. Također je u svih ispitanika s dijagnozom cerebralne paralize utvrđeno prisustvo najčešćih mutacija povezanih s ASA pseudodeficijenzijom (N350S, 1524+95 A>G) i metakromatskom leukodistrofijom (P426L). Promjene aktivnosti i kinetičkih parametara leukocitne ASA u cerebralnoj paralizi najvjerojatnije su posljedica snižene koncentracije enzima; moguće je da nađene mutacije barem djelomično doprinose zapaženim promjenama.

Ključne riječi: arilsulfataza A, kinetički parametri, aktivnost enzima, mutacije u genu za arilsulfatazu A, cerebralna paraliza

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