Short Communication

Autistic children exhibit distinct plasma amino acid profile

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Received 01 August 2013; revised 11 September 2013

In order to ascertain whether autistic children display characteristic metabolic signatures that are of diagnostic value, plasma amino acid analyses were carried out on a cohort of 138 autistic children and 138 normal controls using reverse-phase HPLC. Pre-column derivatization of amino acids with phenyl isothiocyanate forms phenyl thio-carbamate derivates that have a λmax of 254 nm, enabling their detection using photodiode array. Autistic children showed elevated levels of glutamic acid (120 ± 89 vs. 83 ± 35 µmol/L) and asparagine (85 ± 37 vs. 47 ± 19 µmol/L); lower levels of phenylalanine (45 ± 20 vs. 59 ± 18 µmol/L), tryptophan (24 ± 11 vs. 41 ± 16 µmol/L), methionine (22 ± 9 vs. 28 ± 9 µmol/L) and histidine (45 ± 21 vs. 58 ± 15 µmol/L). A low molar ratio of (tryptophan/large neutral amino acids) × 100 was observed in autism (5.4 vs 9.2), indicating lesser availability of tryptophan for neurotransmitter serotonin synthesis. To conclude, elevated levels of excitatory amino acids (glutamate and asparagine), decreased essential amino acids (phenylalanine, tryptophan and methionine) and decreased precursors of neurotransmitters (tyrosine and tryptophan) are the distinct characteristics of plasma amino acid profile of autistic children. Thus, such metabolic signatures might be useful tools for early diagnosis of autism.

Keywords: Autism, Glutamate, Asparagine, Tryptophan, Methionine

Autism is a pervasive developmental disorder that usually presents prior to three years of age and is characterized by impairment in three domains: social interaction, communication and imaginative or creative play$. The incidence of autism spectrum disorders is reported to be 1 in 88 worldwide and 1/500 in India$. The prevalence of autism is more among boys than girls and occurs with high frequency in nuclear families and families with moderate to high socio-economic status$²,³.

The etiology of autism is complex and involves both genetic and environmental factors. High prevalence of autism among siblings and monozygotic twins suggests strong genetic basis for autism$. However, phenotypic heterogeneity among the members of the same family or pedigree indicates polygenic mode of inheritance and influence of environmental exposures. Co-morbid conditions, such as Rett syndrome, fragile X syndrome, phenylketonuria, adenylsuccinate lyase deficiency, dihydropyrimidine dehydrogenase deficiency and 5-nucleotidase hyperactivity account only for <10% of cases. There have been several attempts to find a common cause or biochemical marker for autism in remaining 90% cases. However, majority of these studies have used very small sample size and have been focused on autism spectrum disorders, rather than autism alone.

In addition to behavioral abnormalities, autistic persons have high prevalence of gastrointestinal disease, which may adversely affect the absorption of certain dietary components. Certain aminoacids serve as the biosynthetic precursors for the neurotransmitters, such as serotonin, dopamine and norepinephrine. Autistic behavior has been attributed to functional imbalance among these neurotransmitters. High urinary levels of homovanillic acid have been reported in autistic children. Essential amino acid deficiencies, elevated levels of excitatory amino acids (glutamic acid and aspartic acid), low glutamine and methionine levels, decreased synthesis of serotonin in frontal cortex and thalamus have been reported in autistic children.

In view of the metabolic abnormalities being prevalent in autism and paucity of substantial studies from India, in this study, we have aimed to investigate the plasma amino acids in a new cohort of autism cases and controls with increased sample size, in order to provide better understanding of the metabolic basis for autism.
Materials and Methods

Subjects

The study population consisted of 138 autistic children (120 males and 18 females) and 138 non-autistic control children (120 males and 18 females) matched for age, gender, ethnicity and geographical area. The mean age of cases and controls was 4.4 ± 1.7 yrs and 4.4 ± 1.6 yrs, respectively (age group ranging from 2 to 10 yrs). Recruitment took place during the period of 2001-2006. The diagnosis of autism was made in a two-tier system: i) by evaluating all the subjects for ‘Diagnostic and Statistical Manual of Mental Disorders’ (DSM-IV) criteria, and ii) by Autism Behavior Checklist (ABC) scoring (based on 57 questions). The scores for five different domains, namely sensory stimuli, relating, body and object use, language and social self-help were obtained based on the parental interview. Children with ABC score >68 were enrolled as cases and control children had ABC scoring < 21. The details of scoring under each domain are described elsewhere.

In addition, language function was assessed in children using 'Receptive Expressive, Emergent Language Scale' and Developmental Assessment using different tests including the Gesell's developmental test, the Raven's colored matrices, the Vineland social maturity test and the Binet Kamat test for general mental ability). The developmental quotient (DQ) in the autistic group ranged from 30 to 80 and most of them had mild-to-moderate developmental delay. DQ of control children was >80. Informed consent was obtained from parents or guardian of each participant before enrollment. Ethical clearance for this study was obtained from the Bioethical Committee of Center for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad.

Exclusion criteria

Subjects suffering from a neurological, inflammatory, endocrine or clinically significant chronic disease, immuno-compromized subjects, subjects receiving psychoactive drugs or drugs with known or potential interaction with plasma amino acids and with an active seizure disorder were excluded from the study. All the subjects underwent genetic and biochemical investigations and were excluded when found positive for fragile X syndrome, chromosomal anomalies and classical aminoacidopathies like phenylketonuria. The samples were obtained immediately after the initial diagnosis and hence subjects were not on any special diet during the time of analysis. In order to reduce the dietary influence on analysis, fasting blood samples were collected.

Estimation of plasma amino acids using reverse-phase HPLC

Whole blood samples were collected into EDTA-containing evacuated tubes and immediately chilled on ice before being centrifuged at 4000 × g for 10 min at 4°C. Plasma aliquots were transferred into cryostat tubes and stored at −70°C until the analysis. To precipitate proteins, 50 µl of freshly prepared 10% trichloro acetic acid was added to 50 µl of plasma and mixed well. After centrifugation at 18000 × g for 15 min at 4°C, the supernatant fluid was filtered through a 0.2 µm nylon membrane filter (Millipore). Aliquots of 12.5 µl of standard or filtered sample were taken in respective tubes and equal volumes of internal standard L-methionine sulfone were added. The resulting mixtures were allowed to dry in vacuum. 10 µl of drying solution (2:2:1 mixture of methanol: 1 M sodium acetate: triethylamine) was added in each tube, mixed well and dried in vacuum.

To each tube, 20 µl of derivatization solution (7:1:1:1 mixture of methanol: triethylamine: HPLC grade water: phenyl isothiocyanate) was added, mixed well, incubated for 20 min at ambient temperature and dried in vacuum. Again, to each tube, 100 µl of diluent (4 mM disodium hydrogen phosphate adjusted to pH 7.4 with 10% phosphoric acid, 4% acetonitrile v/v) was added and agitated carefully to avoid sample loss. Each sample was transferred to a Model 712 WISP vial equipped with a limited volume insert. All the samples were analyzed within a day of derivatization, in order to minimize the possibility of degradation. Aspartic acid and asparagine ratio was calculated in both cases and controls to check for amino acid degradation and found to be statistically insignificant.

HPLC elution and photodiode array (PDA) detection were carried out using Waters HPLC system. Inertsil ODS-3V, 5 µm, 4.6 × 250 mm column (GL Sciences Inc.) was used as a stationary phase. Gradient-based resolution was carried out using Eluent A (140 mM sodium acetate trihydrate, 5% v/v triethylamine, adjusted to pH 6.4 with glacial acetic acid, 6% acetonitrile, v/v) and Eluent B (60% acetonitrile). 20 µl sample was injected using autosampler. Column heater temperature was kept at 46°C and detector was set at 254 nm with a run time...
of 87 min. The unknown concentrations of plasma metabolites were calculated from peak areas and standard calibration curves using Millennium software of Waters HPLC system.

**Statistical analysis**

Levels of plasma amino acids were considered as continuous variables. These variables for each parameter were tabulated as two data sets: one corresponding to cases and another to controls. Student’s t-test was performed and mean and SD values were obtained. To correct for non-parametric distribution, Mann Whitney U test was also performed and Z and P values were obtained. Both these tests were performed using Graphpad and Vassarstats softwares. The 5th, 50th and 95th percentiles were calculated for cases and controls for each parameter. The 5th percentile of controls was taken as lower cut-off value and the 95th percentile of controls was taken as higher cut-off value. Any value above the higher cut-off (elevated) or below the lower cutoff (depleted) was considered abnormal.

**Results and Discussion**

**Excitotoxicity due to elevated glutamate and asparagine**

As shown in Table 1, significant differences were observed between the aminoacid profiles of cases and controls. Excitatory amino acids i.e. glutamic acid (120 ± 89 vs. 83 ± 35 µmol/L, P<0.01), asparagine (85 ± 37 vs. 47 ± 19 µmol/L, P <0.0001) levels were significantly higher in autistic cases, compared to controls. Elevated glutamate and asparagine levels were observed in 26% and 54% autistic children, respectively. Our findings in demonstrating elevated glutamate and asparagine levels in autistic children were in agreement with the previous reports\(^{14,15,17}\).

N-acetylaspartylglutamate (NAAG), a neuropeptide is hydrolyzed to N-acetyl aspartate and glutamate in the presence of N-acetylated-α-linked acidic dipeptidase (NAALADase). The released glutamate binds to different receptors (NMDA, AMPA, Kainite, metabotropic), resulting in their activation. Glutamate is removed from the synapse after the activation and is carried to astrocytes by glutamate transporter 1 (GLT1) and glutamate aspartate transporter (GLAST), where it is stored as glutamine via the action of glutamine synthetase\(^{18}\). This is then transported back to the presynaptic neurons and reconverted to glutamate via glutaminase.

Alterations in the glutamate transporter, or glutaminase or glutamine synthetase activity would potentially alter the glutamine/glutamate ratio. Elevated levels of glutamate (brain) can have adverse effects due to increased influx of calcium ions\(^{19}\). Excessive intracellular calcium stimulates a series of enzymes, such as protein kinase C, calcium/ calmodulin-dependent protein kinase II, phospholipases.

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<th>Amino acid profile in autism cases and non-autistic controls</th>
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All results are shown as mean (SD) and in µmol/L; CAA1: sum of tyrosine, phenylalanine, valine, leucine and isoleucine; CAA2: sum of tryptophan, phenylalanine, valine, leucine and isoleucine; *= statistically significant; \(e\): elevated levels >95th percentile of non-autistic children; \(d\): decreased levels <5th percentile of non-autistic children
proteases, phosphatases, nitric oxide synthase, endonucleases, ornithine decarboxylase, xanthine oxidase, which are involved in normal neuronal development and function\textsuperscript{19}. Excessive activation of these enzymes may contribute to injury to the cell membrane, cytoskeleton, or DNA. Nitric oxide and peroxynitrite generated by the action of serine protease tissue plasminogen activator and nitric oxide synthase can damage the blood brain barrier, thereby altering its permeability\textsuperscript{20}. Altered permeability of blood brain barrier may allow the efflux and influx of glutamate from brain to blood and vice-versa. Asparagine can also bind to NMDA receptors and cause calcium flux\textsuperscript{21}. Recently, we have reported protective role of NAALADase C1561T polymorphism against autism, which is attributed to steric hindrance to NAAG binding\textsuperscript{22}.

**Tryptophan depletion**

The current study showed lower levels of phenylalanine and tryptophan in autistic children (24 ± 11 vs. 41 ± 16 μmol/L, P<0.0001). These two amino acids are precursors for neurotransmitters dopamine and serotonin, respectively. Tryptophan to competing amino acids (phenylalanine, tyrosine, isoleucine, leucine and valine) ratio was significantly lower in cases, compared to controls (5.4 ± 2.7 vs. 9.2 ± 3.9, P<0.0001). Decreased tryptophan, methionine, histidine and phenylalanine levels were observed in 48%, 28%, 52% and 33% autistic children, respectively.

Tryptophan and other large neutral amino acids compete for brain serotonin synthesis. Low tryptophan signals low brain serotonin synthesis. Earlier studies have shown low tryptophan levels in autistic children\textsuperscript{23,24}, while deterioration in the behavior of autistic children is reported after short-term tryptophan depletion\textsuperscript{25}. In the normal developmental process in humans, a period of high brain serotonin synthesis capacity is noticed until the age of 5 yrs. This may be disrupted in autistic children\textsuperscript{26}.

As vitamin B6 is important for conversion of tryptophan to serotonin, deficiency of tryptophan or/and B6 can produce a biochemical imbalance in the brain causing mood and behavioral changes. Positron emission tomography study on Rhesus monkey has shown regulatory role of pyridoxine in serotonin synthesis\textsuperscript{27}. Improvement in language abilities, affectivity and response to behavior modification therapy is also reported in three out of eight children supplemented with vitamin B6\textsuperscript{28}.

**Essential amino acid deficiencies**

In the current study, lower methionine (22 ± 9 vs. 28 ± 9 μmol/L, P<0.0001), histidine (45 ± 21 vs. 58 ± 15 μmol/L, P <0.0001), phenylalanine (45 ± 20 vs. 59 ± 18 μmol/L, P<0.0001) levels were observed in cases, compared to controls. Earlier study has also observed low methionine levels and low S-adenosyl methionine to S-adenosyl homocysteine ratio in autism and has attributed the pathophysiology of autism to the decreased cellular methylation\textsuperscript{29}. Methionine being a precursor for S-adenosyl methionine, a universal methyl donor has a major impact on methylation of DNA, proteins and neurotransmitters. Gastric hypochlorhydria and gastric dysfunction caused due to intrinsic factor or B12 deficiency correlate positively with low methionine levels\textsuperscript{30,31}.

Autistic children enrolled in this study had higher frequency of methylene tetrahydrofolate reductase (MTHFR) C677T (T-allele frequency: 16.3 vs. 6.5%) and methionine synthase reductase (MTR) A66G (G-allele frequency: 87.3% vs. 79%) polymorphisms, compared to controls\textsuperscript{16}. These polymorphisms might interfere with methionine synthesis by decreasing the availability of co-substrate (5-methyl tetrahydrofolate) and cofactor (methylcobalamin) necessary for remethylation of homocysteine to methionine.

Histidine was found to be significantly low in autistic children. It is important in the histamine release and is an important mediator in inflammatory response and initiates gastric secretion. Histamine requirement in children is high as the digestive enzymes are still immature. Low histamine levels also lead to hypochlorhydria and gastrointestinal disturbances\textsuperscript{32}. Hypochlorhydria, in turn, leads to intrinsic factor deficiency\textsuperscript{33}.

In conclusion, autistic children exhibited distinct amino acid patterns, such as glutamatergic transmission, decreased neurotransmitter synthesis, essential amino acid deficiencies, which might be useful as diagnostic tools of autism. Future studies are warranted to see whether supplementation with vitamin B6 and methylcobalamin is beneficial for autistic children.

**Acknowledgements**

The research was supported by the core grant provided by Department of Biotechnology (DBT), India. We thank all the families who participated in this study.
References