Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism\textsuperscript{1,2}

S Jill James, Paul Cutler, Stepan Melnyk, Stefanie Jernigan, Laurette Janak, David W Gaylor, and James A Neubrander

ABSTRACT

Background: Autism is a complex neurodevelopmental disorder that usually presents in early childhood and that is thought to be influenced by genetic and environmental factors. Although abnormal metabolism of methionine and homocysteine has been associated with other neurologic diseases, these pathways have not been evaluated in persons with autism.

Objective: The purpose of this study was to evaluate plasma concentrations of metabolites in the methionine transmethylation and transsulfuration pathways in children diagnosed with autism.

Design: Plasma concentrations of methionine, $S$-adenosylmethionine (SAM), $S$-adenosylhomocysteine (SAH), adenosine, homocysteine, cystathionine, cysteine, and oxidized and reduced glutathione were measured in 20 children with autism and in 33 control children. On the basis of the abnormal metabolic profile, a targeted nutritional intervention trial with folinic acid, betaine, and methylcobalamin was initiated in a subset of the autistic children.

Results: Relative to the control children, the children with autism had significantly lower baseline plasma concentrations of methionine, SAM, homocysteine, cystathionine, cysteine, and total glutathione and significantly higher concentrations of SAH, adenosine, and oxidized glutathione. This metabolic profile is consistent with impaired capacity for methylation (significantly lower ratio of SAM to SAH) and increased oxidative stress (significantly lower redox ratio of reduced glutathione to oxidized glutathione) in children with autism. The intervention trial was effective in normalizing the metabolic imbalance in the autistic children.


KEY WORDS Autistic disorder, biomarkers, oxidative stress, methylation, methionine, $S$-adenosylmethionine, $S$-adenosylhomocysteine, adenosine, cysteine, glutathione

INTRODUCTION

Autism is a neurodevelopmental disability that is usually diagnosed before age 3 y and is characterized by deficits in social reciprocity and in language skills that are associated with repetitive behaviors and restricted interests (1). In addition to behavioral impairment, autistic persons have a high prevalence of gastrointestinal disease and dysbiosis (2), autoimmune disease (3), and mental retardation (4). Autism also affects many more males than females, occurring at a ratio of 4:1. A significant role for genetics in the etiology of the autistic disorder is supported by a high concordance of autism between monozygotic twins and increased risks among siblings of affected children and of autistic symptoms associated with several heritable genetic diseases [see: Online Mendelian Inheritance in Man (OMIM) #209850 (autism; 5)]. Autism has been reported to be a comorbid condition associated with Rett syndrome (5), fragile X (6), phenylketonuria (7), adenylosuccinate lyase deficiency (8), dihydroy- pyrimidine dehydrogenase deficiency (9), and 5\textsuperscript{-}nucleotidase hyperactivity (10); however, these genetic diseases account for <10\% of cases of autism. Nonetheless, the association of autism with genetic deficits in specific enzymes suggests the possibility that the genetic component of primary autism could be expressed as a chronic metabolic imbalance that impacts normal neurodevelopment and immunologic function. The possibility that autism has a metabolic phenotype is less widely accepted but has been supported by several small studies (9, 11–14).

The current study was prompted by the serendipitous observation in a previous study that the metabolic profiles of dizygotic twins—one with Down syndrome and one with autism—were virtually identical with respect to methionine cycle and transsulfuration metabolites (15). Down syndrome, or trisomy 21, is a complex genetic and metabolic disease due to the presence of 3 copies of chromosome 21 and associated with an increased frequency of autism (16). In our previous study, children with Down syndrome had lower concentrations of metabolites in the methionine cycle and significantly lower glutathione concentrations than did control children (15).

The methionine cycle involves the regeneration of methionine via the vitamin B-12–dependent transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine in the methionine synthase reaction. Methionine may then be activated by methionine adenosyltransferase to form $S$-adenosylmethionine (SAM), the primary methyl donor for most cellular methytransferase reactions including the methylation of DNA, RNA, proteins, phospholipids,
and neurotransmitters (Figure 1). The transfer of the methyl group from SAM to the various enzyme-specific methyl acceptors results in the formation of S-adenosylhomocysteine (SAH). The reversible hydrolysis of SAH to homocysteine and adenosine (SAHH) reaction completes the methionine cycle. Adenosine is further metabolized by adenosine kinase for purine synthesis or catabolized by adenosine deaminase (ADA). Homocysteine may be permanently removed from the methionine cycle by irreversible conversion to cystathionine by vitamin B-6-dependent cystathionine β-synthase (CBS). Cystathionine is converted to cysteine, which is the rate-limiting amino acid for the synthesis of the tripeptide glutathione (Glu-Cys-Gly). THF, tetrohydrofolate; 5-CH₂THF, 5-methyltetrahydrofolate; SAHH, SAH hydrolase.

SUBJECTS AND METHODS

Study participants

The participants in the metabolic study were 20 autistic (± SD age: 6.4 ± 1.5 y) and 33 control (age: 7.4 ± 1.3 y) children. The diagnosis of autism was based on the criteria for autistic disorder as defined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and by a diagnostic interview conducted by a developmental pediatrician. Of the 20 autistic children, all were white, 14 were boys, 6 were girls, 19 were diagnosed with regressive autism, and 1 had infantile autism. Most of these children were impaired in speech and socialization skills and exhibited symptoms of gastrointestinal distress; before the study, 16 were taking a multivitamin and mineral supplement containing 400 µg folic acid and 3 µg vitamin B-12.

None of the autistic children were taking prescribed medicines, such as valproic acid or anticonvulsants, that might have affected methionine metabolism. A quantifiable diet questionnaire was not administered as part of this study; thus, specific dietary differences within and between groups cannot be determined. The control subjects in the metabolic study were healthy white US children with no history of chronic disease or autism who had participated in a similar baseline study of children with Down syndrome (15). Control children took over-the-counter vitamin supplements and were not taking medications known to interfere with methionine metabolism. Exclusion criteria for both groups included a diagnosis of malnutrition, the presence of active infection, or known genetic disease.

The protocol and informed consent for this study were reviewed and approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. The details of the study were explained to the parents of the participating children, and written informed consent was obtained from the parents.

Study design

The metabolic study consisted of 3 parts. In the first component, baseline concentrations of plasma metabolites in the methionine cycle and transsulfuration pathway were measured in 20 autistic children and compared with plasma concentrations in 33 control children to establish whether the metabolic profile of the autistic children differed significantly from that of the control children. In the second component, based on the observed abnormalities in plasma metabolites, a subset of 8 autistic children were given oral supplements of 800 µg folinic acid and 1000 mg betaine (anhydrous trimethylglycine) twice a day in an attempt to improve the metabolic profile; this is referred to as intervention 1. After 3 mo on this regimen, blood samples were again taken and the metabolite concentrations were compared with baseline concentrations of each metabolite. In the third component, the same subset of 8 children were given an injectible form of methylcobalamin (75 µg/kg) twice a week in addition to the oral folinic acid and betaine for an additional month; this is referred to as intervention 2. Each child served as his or her own control for the intervention study.

Nutritional supplements

USP-grade folinic acid was obtained from Douglas Laboratories (Pittsburgh) or Thorne Research, Inc (Dover, ID) and was given twice a day as 800 µg oral powder in juice. Betaine (trimethylglycine, USP grade) was purchased from Life Extension Foundation (Fort Lauderdale, FL) and given twice a day as 1000 µg oral powder in juice. USP methylcobalamin was obtained from Hopewell Pharmaceuticals (Hopewell, NJ) or Unique Pharmaceuticals (Temple, TX) as an injectible liquid and given subcutaneously at a dose of 75 µg/kg twice a week.

Sample treatment and HPLC method

Fasting blood samples were collected into EDTA-containing evacuated tubes (B-D Biosciences, Dallas) 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 −80 °C until extraction and HPLC quantification. For determination of methionine, total homocysteine, cysteine, and...
total glutathione (tGSH) concentrations, 50 μL of a freshly prepared solution of 1.43 mmol sodium borohydride/L containing 1.5 μmol EDTA/L, 66 mmol NaOH/L, and 10 μL isomyl alcohol was added to 200 μL plasma to reduce all sulfhydryl bonds. The samples were incubated at 40°C in a shaker for 30 min. To precipitate proteins, 200 μL ice-cold 10% metaphosphoric acid was added and mixed well, and the sample was incubated for an additional 10 min on ice. After centrifugation at 18,000 × g for 15 min at 4°C, the supernatant fluid was filtered through an 0.2-μm nylon membrane filter (PGC Scientific, Frederick, MD), and a 20-μL aliquot was injected into the HPLC system. For measurement of SAM, SAH, adenosine, cystathionine, and oxidized glutathione (GSSG) concentrations, 100 μL of 10% metaphosphoric acid was added to 200 μL plasma to precipitate protein; the solution was mixed well and incubated on ice for 30 min. After centrifugation for 15 min at 18,000 g at 4°C, supernatant fluids were passed through an 0.2-μm nylon membrane filter, and 20 μL was injected into the HPLC system.

The details of the method for HPLC elution and electrochemical detection were described previously (18, 19). The separation of metabolites was performed by using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse-phase 5-μm C18 column (4.6 × 150 mm; MCM Inc, Tokyo) obtained from ESA Inc (Chemsford, MA). A 20-μL aliquot of plasma extract was directly injected onto the column by using a Beckman Autosampler (model 507E; Beckman Instruments, Irvine, CA). All plasma metabolites were quantified by using model 5200A Coul Cher II and CoulArray electrochemical detection systems equipped with a dual analytic cell (model 5010), a 4-channel analytic cell (model 6210), and a guard cell (model 5020) (all: ESA Inc). The unknown concentrations of plasma metabolites were calculated from peak areas and standard calibration curves with the use of HPLC software.

Statistical analysis

Metabolic data are presented as means ± SDs. Statistical differences in plasma metabolites between case and control children were ascertained by using the Student’s t-test with significance set at 0.05. One-way analysis of variance was performed to ascertain whether differences existed between plasma metabolite concentrations at the 3 time points: baseline (no intervention), after intervention 1 (folic acid and betaine), and after intervention 2 (folic acid, betaine, and methylcobalamin). Individual metabolites at baseline were subsequently compared with those after intervention 1 and intervention 2 by using the paired Student’s t-test with the Bonferroni correction. Statistical analyses were accomplished with the use of SIGMASTAT software (version 2.0; Systat Software Inc, Richmond, CA).

RESULTS

Baseline methionine cycle and transsulfuration pathway metabolites

The baseline concentrations of metabolites in the methionine cycle and in the transsulfuration pathway were significantly different between the autistic children and the control children. Within the methionine cycle, plasma concentrations of methionine, SAM, and homocysteine were significantly lower and SAH and adenosine concentrations were significantly higher than those in the control children (Table 1). The ratio of SAM to SAH was almost 50% lower in the autistic children than in the control children. The significant reductions in plasma cystathionine and cysteine concentrations observed in the autistic children (Table 1) were consistent with a decrease in CBS-mediated transsulfuration. Associated with the low mean plasma cysteine concentration was a significant decrease in tGSH concentrations. GSSG was increased almost twofold, and tGSH:GSSG was reduced by 70%.

Supplementation with folic acid and betaine (intervention 1)

A subset of 8 autistic children participated in an intervention trial designed to improve their metabolic profile. Oral supplementation with 800 μg folic acid and 1000 mg betaine, both given twice a day, was maintained for a period of 3 mo (intervention 1), and a second blood sample was drawn. Relative to baseline concentrations, mean plasma methionine, SAM, homocysteine, cystathionine, cysteine, and tGSH concentrations and SAM:SAH and tGSH:GSSG in these 8 children were higher (Table 2). In addition, the high SAH and adenosine concentrations observed at baseline decreased with the betaine and folic acid supplements during intervention 1. The mean concentrations of methionine, SAM, SAH, adenosine, and homocysteine were not statistically different from those in the control children, which indicated that intervention with folic acid and betaine had brought these methionine cycle metabolites into the normal range. Although supplementation was effective in normalizing the methionine cycle metabolites to the concentrations in the control subjects, the intervention significantly improved but did not normalize tGSH or GSSG concentrations or tGSH:GSSG.

Supplementation with folic acid, betaine, and methyl vitamin B-12 (intervention 2)

For intervention 2, an injectible form of methylcobalamin (75 μg/kg) was added to the folic acid and betaine regimen for a period of 1 mo, after which the third blood sample was taken for
HPLC analysis. The addition of injectable methylcobalamin (intervention 2) did not alter the mean concentrations of methionine, SAM, SAH, or homocysteine beyond the alterations induced by the intervention with folic acid and betaine (Table 2). However, relative to intervention 1, the addition of injectable methylcobalamin further decreased the concentrations of adenosine and GSSG and further increased the concentrations of methionine, cysteine, and tGSH and SAM:SAH and tGSH:GSSG.

DISCUSSION

Autism is a complex neurodevelopmental disorder that is thought to involve an interaction between multiple, variable susceptibility genes (21), epigenetic effects (22), and environmental factors (23). The apparent increase in the diagnosis of autism-spectrum disorders from 4–5 in 10 000 children in the 1980s to 30–60 in 10 000 children in the 1990s has raised great concern (24–27). This increased prevalence of autism has enormous future public health implications and has stimulated intense research into potential etiologic factors and candidate genes. Because abnormal folate metabolism and low glutathione concentrations have been reported in other neurologic disorders, including Alzheimer disease, Parkinson disease, schizophrenia, and Down syndrome (15, 28–31), we measured the concentrations of methionine methylation and transsulfuration metabolites in a cohort of autistic children.

The concentrations of metabolites among the control children in this study were within the range of values previously found in several studies (32–34). The observed imbalance in methionine and homocysteine metabolism in the autistic children is complex and not easily explained by perturbation of a single pathway or isolated genetic or nutritional deficiency. Within the methionine cycle, significant decreases in plasma concentrations of methionine, SAM, and homocysteine were associated with significant increases in adenosine and SAH. The low methionine and SAM concentrations would suggest a reduction in methionine synthase activity; however, the observed decrease in homocysteine does not fit that interpretation. The data may be best explained by oxidative inactivation of methionine synthase in combination with a decrease in SAH hydrolase activity secondary to the increase in adenosine (35, 36). Adenosine binds to the active site of SAH hydrolase, and increased concentrations of adenosine have been shown to reduce SAHH activity (36, 37). A combined enzyme deficit would also be consistent with the observed decrease in SAM and increase in SAH concentrations. In this case, the decrease in homocysteine concentrations would reflect an adenosine-mediated decrease in SAH hydrolysis and homocysteine synthesis. The functional consequence of an increase in SAH is product inhibition of most cellular methyltransferases (38). Low methionine and SAM concentrations in combination with increased SAH and adenosine concentrations were shown previously to be associated with reduced cellular methylation capacity (39). The twofold decrease in SAM:SAH also suggests an impaired capacity in these autistic children for cellular methylation.

The metabolic pattern observed in the transsulfuration pathway may provide a more cohesive explanation for the unusual imbalance in methionine cycle metabolites. Low concentrations of cystathionine, cysteine, and tGSH are consistent with reduced flux through the transsulfuration pathway. Furthermore, the significant increase in GSSG disulfide and the 67% decrease in tGSH:GSSG indicate chronic oxidative stress. Within the methionine cycle, methionine synthase, betaine homocysteine methyltransferase, and methionine adenosyltransferase are all redox-sensitive enzymes that are down-regulated by oxidative stress (40–42). A decrease in methionine- and SAM-regulated CBS activity would increase the requirement for cysteine, effectively making it an essential amino acid in these children. Because cysteine is the rate-limiting amino acid for glutathione synthesis, its decrease is consistent with low concentrations of glutathione (43, 44). The remarkably consistent decrease in cysteine and glutathione concentrations and tGSH:GSSG in the autistic children suggests an increased vulnerability to oxidative stress.

The genetic or environmental factors (or both) that would initiate oxidative stress and abnormal metabolic profiles in the autistic children are not clear. It is possibly relevant that, in autistic children, decreased activity of adenosine deaminase and

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 8)</th>
<th>Intervention 1 (n = 8)</th>
<th>Intervention 2 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine (μmol/L)</td>
<td>19.2 ± 3.5</td>
<td>25.7 ± 3.6</td>
<td>30.9 ± 7.7</td>
</tr>
<tr>
<td>SAM (nmol/L)</td>
<td>75.5 ± 5.0</td>
<td>112.9 ± 20.8</td>
<td>101.6 ± 20.5</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>27.6 ± 6.1</td>
<td>16.9 ± 6.5</td>
<td>14.3 ± 7.5</td>
</tr>
<tr>
<td>SAM:SAH</td>
<td>2.9 ± 0.8</td>
<td>7.4 ± 4.1</td>
<td>8.9 ± 4.5</td>
</tr>
<tr>
<td>Adenosine (μmol/L)</td>
<td>0.30 ± 0.2</td>
<td>0.18 ± 0.04</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>5.4 ± 0.9</td>
<td>6.7 ± 0.7</td>
<td>7.4 ± 1.7</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.10 ± 0.02</td>
<td>0.22 ± 0.08</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>166 ± 11.4</td>
<td>180 ± 11</td>
<td>199.3 ± 15</td>
</tr>
<tr>
<td>tGSH (μmol/L)</td>
<td>4.0 ± 0.7</td>
<td>5.0 ± 0.9</td>
<td>6.7 ± 1.6</td>
</tr>
<tr>
<td>Oxidized glutathione (nmol/L)</td>
<td>0.59 ± 0.2</td>
<td>0.38 ± 0.1</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>tGSH:GSSG</td>
<td>7.5 ± 2.3</td>
<td>13.8 ± 3.9</td>
<td>28.7 ± 7.1</td>
</tr>
</tbody>
</table>

1 All values are x ± SD. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; tGSH, total glutathione; GSSG, oxidized glutathione. Intervention 1: 800 μg folinic acid and 1000 mg betaine were administered twice a day from immediately after the baseline blood draw for a period of 3 mo; intervention 2: subcutaneous injection of 75 μg methylcobalamin/kg was added to folic acid and betaine supplementation for an additional month.

2 Intervention 1 compared with baseline.

3 Intervention 2 compared with intervention 1.
increased frequency of adenosine deaminase polymorphisms have been shown to be associated with low adenosine deaminase activity (14, 45). The observed increase in adenosine could be due to either an inhibition of adenosine kinase or an increase in 5-nucleotidase, both of which have been shown to occur with oxidative stress (46, 47). Elevated intracellular adenosine has been shown to inhibit glutathione synthesis (48, 49). Alternatively, a genetic predisposition to environmental agents or conditions that promote oxidative stress could contribute to the abnormal metabolic profile observed in the autistic children.

The targeted nutritional intervention trial in a subset of the autistic children was specifically designed to increase methionine concentrations (intervention 1). Betaine homocysteine methyltransferases provides a folate–vitamin B-12–dependent pathway in the liver and kidney to remethylate homocysteine to methionine (17). Supplemental betaine (trimethylglycine) has been shown to up-regulate betaine homocysteine methyltransferase expression and activity to increase methionine synthesis (50). Folinic acid (5-formyl tetrahydrofolate) was used rather than folic acid because the former is absorbed as the reduced metabolite and can enter folate metabolism as 5,10-methylene tetrahydrofolate, thereby reducing the possibility of promoting a folate trap (51, 52). As shown in Table 2, the intervention with betaine and folic acid was successful in bringing all the metabolites within the methionine cycle into the normal range and simultaneously improving significantly the metabolites in the transsulfuration pathway. The increase in methionine, SAM, and homocysteine concentrations and the decrease in adenosine and SAH concentrations suggested that the intervention stimulated an increased flux through the methionine cycle. In addition, the significant increase in cystathionine concentrations suggests that the supplements were effective also in increasing CBS activity, most likely because of up-regulation by the increase in SAM. The associated increases in cysteine and glutathione indicate that transsulfuration to glutathione was enhanced by the supplements. The decrease in adenosine is consistent with a concomitant release of SAH inhibition and decrease in SAH and, possibly, the release of a bottleneck in methionine cycle turnover. The mechanism for the decrease in adenosine concentrations, however, is not clear. One possibility is that the increase in cysteine or glutathione concentration (or both) relieved the need for adenosine as a protective factor against oxidative damage (53, 54).

The intervention of injectible methylcobalamin to the protocol (intervention 2) was based on empirical observations of clinical improvement in speech and cognition (by JAN) and the possibility that it might enhance methionine synthase activity under conditions of oxidative stress by replacing oxidized inactive coenzyme B-12 [cob(II)alamin] or by posttranslational up-regulation of methionine synthase, or both (55, 56). One month after the addition of methylcobalamin, the methionine concentrations were within the control range (Table 1), and further improvements in adenosine and SAH concentrations and SAM: SAH were observed. Unexpectedly, and perhaps most significantly, the addition of methylcobalamin reduced the concentrations of inactive GSSG and increased the GSH concentrations and GSH:GSSG so that they were not different from those in the control children (Table 1). These positive changes in the glutathione redox profile most likely reflect the increase in cysteine as the rate-limiting amino acid for glutathione synthesis (44). Of note, there is a higher demand for cysteine (and, indirectly, methionine) for de novo glutathione synthesis during chronic oxidative stress (43). Low antioxidant enzyme activity in autistic children has been reported in 3 recent studies (57–59) that provide additional support for oxidative stress as a part of the etiology of autism. If the decreases in plasma methionine, cysteine, and glutathione concentrations in autistic children observed in the current study are confirmed in a larger study, low concentrations of these thiol metabolites could provide metabolic biomarkers for autism.

Although clinical improvements in speech and cognition were noted by the attending physician (PC), they were not measured in a quantifiable manner and are therefore not reported here. Specific dietary differences between groups could have contributed to our results, but we consider it unlikely that uniform dietary differences within the autistic group existed that could have accounted for the remarkably consistent metabolic alterations. Increased frequency of common polymorphisms in these pathways may have contributed to the observed metabolic phenotype, and studies of that subject, as well as studies to quantify clinical improvement, are currently underway. Our attempts to interpret these preliminary metabolic findings are clearly speculative, and a better understanding of the abnormal one-carbon metabolism in these children will require additional research efforts. Nonetheless, the ability to correct the metabolic imbalance with targeted nutritional intervention implies that certain aspects of autism may be treatable.

Nineteen of the 20 children participating in the study were diagnosed with “regressive” autism (apparently normal development until regression into autism between ages 1.5 and 3 y). On the basis of their abnormal metabolic profiles, we hypothesize that an increased vulnerability to oxidative stress (environmental, intracellular, or both) and impaired methylation capacity may contribute to the development and clinical manifestation of regressive autism.

SJJ was responsible for study design, study coordination, interpretation of data, and manuscript writing. PC was responsible for patient recruitment, obtaining supplements, patient compliance, monitoring clinical status, and methylcobalamin injections. SM was responsible for HPLC quantification of plasma metabolites and data collection and interpretation. SJ was responsible for plasma and DNA extraction and data collection and interpretation. LJ was responsible for patient recruitment, study coordinating and consulting, and data interpretation. DWG was responsible for statistical analysis of data. JAN was responsible for initiating the methylcobalamin treatment in autistic patients, providing consultation, and interpreting data. None of the authors has any financial conflict of interest.

REFERENCES


Downloaded from https://academic.oup.com/ajcn/article-abstract/80/6/1611/4690493 by guest on 18 June 2018


50. Park EI, Renduchintala MS, Garrow TA. Diet-induced changes in hepatic betaine-homocysteine methyltransferase activity are mediated by changes in the steady-state level of its mRNA. J Nutr Biochem 1997;8:541–5.


