

BRIEF REPORT

Adverse Effect of Nitrous Oxide in a Child with 5,10-Methylenetetrahydrofolate Reductase Deficiency

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NITROUS OXIDE IRREVERSIBLY OXIDIZES THE COBALT ATOM OF VITAMIN B₁₂, thereby inhibiting the activity of the cobalamin-dependent enzyme methionine synthase (or 5-methyltetrahydrofolate–homocysteine S-methyltransferase; Enzyme Commission code EC 2.1.1.13). Methionine synthase catalyzes the remethylation of 5-methyltetrahydrofolate and homocysteine to tetrahydrofolate and methionine (Fig. 1). Methionine, by way of its activated form, S-adenosylmethionine, is the principal substrate for methylation in many biochemical reactions, including assembly of the myelin sheath, methyl substitutions in neurotransmitters, and DNA synthesis in rapidly proliferating tissues.¹

We report the neurologic deterioration and death of a child anesthetized twice with nitrous oxide before the diagnosis of 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) deficiency (Online Mendelian Inheritance in Man number 236250) was established.² MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate. Sequence analysis of RNA transcripts and genomic DNA from the patient and his family members, together with direct assays of MTHFR activity in fibroblasts, revealed that the enzyme deficiency was caused by a novel MTHFR mutation (1755G→A), which changes the conserved methionine at position 581 of the enzyme to isoleucine; this mutation is coinherited with two other, common MTHFR polymorphisms (677C→T and 1298A→C), each of which is associated with depressed enzyme function.^{3,4} We propose that a nitrous oxide–induced defect of methionine synthase superimposed on an inherited defect of MTHFR caused the patient's death.

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CASE REPORT

Details of the patient's clinical course and biochemical and pathological findings were reported by Beckman et al. in 1987.² In brief, the child appeared normal until three months of age, when a mass in the left leg was noted. It was not known before the patient's surgery that his father and one of his uncles had serum levels of total homocysteine above 20.0 μmol per liter and above 30.0 μmol per liter, respectively (normal range, 5.4 to 13.9). The proband's sibling, who was receiving lifelong therapy with high-dose vitamin B supplements, had a homocysteine level of 4.3 μmol per liter. Neither the father nor the sibling had received nitrous oxide. On preoperative assessment for excisional biopsy of the mass, the patient's physical status was deemed class I according to the American Society of Anesthesiologists criteria (class I denotes good health and class V critical illness). After premedication with atropine and induction of anesthesia with sodium thiopental and succinylcholine, the child's trachea was intubated; anesthesia was maintained with 0.75 percent halothane and 60 percent nitrous oxide in oxygen for 45 minutes.

METHODS

The investigations were carried out with approval of the University of Wisconsin's institutional review board. Written informed consent was obtained from all the participants.

FIBROBLAST CULTURE AND MTHFR ACTIVITY

Fibroblasts were cultured from skin-punch biopsy specimens obtained from both parents and from the patient's stored samples. MTHFR activity was measured at confluence, as previously described.⁶ All the assays were performed in duplicate, with simultaneous assay of a normal control.

PREPARATION AND SEQUENCE ANALYSIS OF GENOMIC DNA

Genomic DNA was isolated from the cultured fibroblasts from the patient and both parents and from either blood or buccal cells from other relatives. Each of the 11 MTHFR exons was amplified from genomic DNA by the polymerase chain reaction (PCR) with the use of newly designed intronic primers.^{7,8} (The sequences of the primers are listed in Supplementary Appendix 1 with the full text of this article at <http://www.nejm.org>.) The patient's and both parents' PCR products were bidirectionally sequenced. A novel mutation in the patient's DNA at nucleotide 1755 (exon 10) and two previously described frequent polymorphisms at positions 677 (exon 4) and 1298 (exon 7) in the MTHFR gene were analyzed in the genomic DNA from the parents and other relatives with the use of the restriction enzymes *Nla*III, *Hinf*I, and *Mbo*II, as previously described.^{3,4} Family members were also screened as previously described for common polymorphisms in the genes encoding enzymes that regulate folate and homocysteine metabolism; these polymorphisms have been implicated in the pathogenesis of neural-tube defects, other congenital anomalies, and cardiovascular and neoplastic disease.⁹ The polymorphisms include those that encode methionine synthase (*MTR*; the polymorphism results in the substitution of glycine for aspartic acid at residue 919),¹⁰ methionine synthase reductase (*MTRR*; the polymorphism results in the substitution of methionine for isoleucine at residue 22),¹¹ and cystathionine β -synthase (*CBS*; the polymorphism is a 68-bp duplication).¹²

RNA ANALYSIS

To evaluate the expression of an intact copy of the predominant 7.2-kb MTHFR isoform,¹³ RNA was

isolated from the patient's cultured fibroblasts. A 2206-bp product containing the entire coding region was amplified by PCR from the complementary DNA (cDNA) transcript and sequenced in full. The 7.2-kb cDNA product was amplified as seven overlapping fragments ranging from 1.0 to 2.2 kb in size, as verified by gel electrophoresis. (The primers used to sequence the cDNA transcript and to amplify the cDNA as overlapping fragments are listed in Supplementary Appendixes 2 and 3, respectively, with the full text of this article at <http://www.nejm.org>.) Bands corresponding to the expected fragment sizes were excised, and the first 300 bases of the 5' and 3' ends were sequenced to allow positive identification of each fragment. Fragments from the patient and an unrelated control were then compared.

RESULTS

ENZYME ACTIVITY IN FIBROBLASTS

The patient's MTHFR activity in two replicates was 0.76 and 0.03 nmol of formaldehyde per milligram of protein per hour (normal mean value, 13.3 ± 4.6 with the use of the current method of measurement⁶), with a simultaneous normal control of 11.52 nmol of formaldehyde per milligram of protein per hour. MTHFR activity in the father and mother (1.8 and 6.1 nmol of formaldehyde per milligram of protein per hour, respectively) was reduced, with a control level of 9.5 nmol of formaldehyde per milligram of protein per hour.

GENOMIC DNA-SEQUENCE ANALYSIS

The patient was found to be heterozygous for a novel mutation, 1755G→A in exon 10, which causes a substitution of isoleucine for methionine at residue 581 (M581I)¹⁴ (GenBank accession number, NM_005957). Restriction-enzyme analysis confirmed the presence of the 1755G→A mutation in the heterozygous patient, his father, his brother, one uncle, and one aunt, but not in 100 control chromosomes. The patient was also heterozygous for a 677C→T mutation in exon 4 (resulting in a substitution of valine for alanine at residue 222) and a 1298A→C mutation in exon 7 (resulting in a substitution of alanine for glutamic acid at residue 429). In addition to being heterozygous for 1755G→A, the father was homozygous (TT) for the 677C→T mutation and homozygous (AA) at 1298A (Fig. 2). The mother was heterozygous for both common polymorphisms and homozygous (wild type) at 1755G. The sibling's haplotype was identical to that of the

patient in all coding regions. The novel mutation at 1755G→A was therefore transmitted to the patient from a paternal chromosome, in *cis* configuration with the 677C→T mutation. Two of the father's four siblings had haplotypes identical to the father's haplotype and were heterozygous for the 1755G→A mutation and homozygous for the 677C→T mutation (Table 1).

We sequenced 25 to 40 bases beyond all intronic boundaries to look for altered splice junctions. There were no substitutions in the 5' and 3' untranslated regions flanking the *MTHFR* gene, within or proximate to a putative binding site for a transcription factor or an actual start site mapped by Gaughan et al.¹³ and Homberger et al.¹⁵ The DNA sequence approximately 550 bp in the 3' direction from the *MTHFR* stop codon and a 400-bp segment encompassing the distal 3'-polyadenylation site contained several polymorphisms, but none at sites with recognized functional significance.

We also performed genomic analysis of the genes encoding methionine synthase, methionine syn-

thase reductase, and cystathionine β -synthase. Genotypes at these loci for all members of the pedigree are provided in Table 1.

RNA ANALYSIS

No size differences were observed among the seven *MTHFR* cDNA fragments, indicating that the patient's fibroblasts expressed an intact *MTHFR* transcript. The 2.2-kb product contained the entire coding region of the transcript and was used to sequence a region beginning 50 bp in the 5' direction from the translational start site and ending 150 bp downstream of the stop codon. This product was of the expected length, and no alternate splicing variants were detected. The entire product was sequenced and compared with the published sequence¹⁴ (GenBank accession number, NM_005957). The presence of the heterozygous common polymorphisms 677C→T and 1298A→C, as well as the heterozygote substitution 1775G→A, was confirmed.

DISCUSSION

The inactivation of methionine synthase by nitrous oxide has been demonstrated with purified enzyme,¹⁶ in cultured cells,^{17,18} in animal models,¹⁹ and in humans.²⁰⁻²² The mean half-time of inactivation is 46 minutes. Residual methionine synthase activity more than 200 minutes after the start of nitrous oxide administration approaches zero.²¹ Mice, pigs, and rats exposed to nitrous oxide have delayed recovery of enzyme activity for periods of four days or more.^{19,23-25} Recovery in cultured cells indicates that nitrous oxide-mediated inhibition is irreversible, with *de novo* synthesis of the enzyme required to restore activity.²⁶

The untoward consequences of nitrous oxide anesthesia in our patient are reminiscent of two recent case reports. In the first, an eight-month-old child had acute neurologic deterioration six days after an 80-minute period of anesthesia with nitrous oxide.²⁷ In the second, a four-month-old child was admitted because of hypotonia, dehydration, and acidosis three weeks after surgery that had involved a 180-minute period of anesthesia with nitrous oxide.²⁸ Both children were found to have severe dietary cobalamin deficiency. These instances of methionine synthase inhibition have a time course and clinical features similar to those observed in our patient but were nonlethal, perhaps because they were elicited after only a single exposure to nitrous oxide.

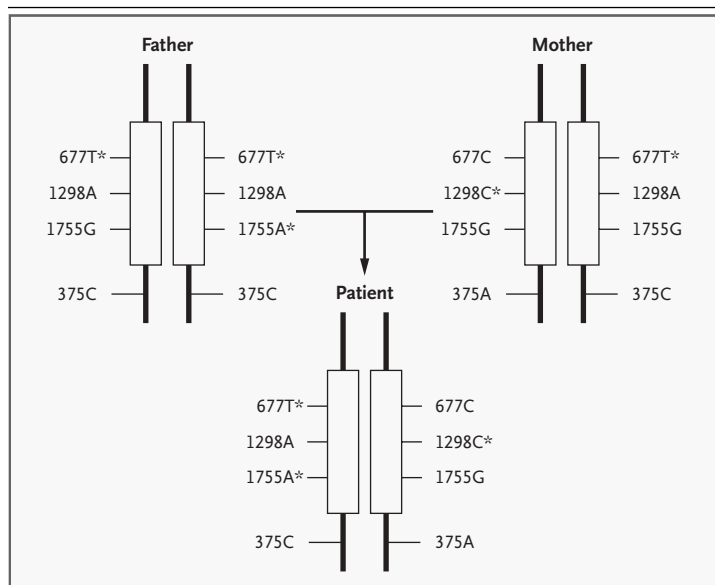


Figure 2. Nucleotide Changes in the *MTHFR* Gene in the Patient and His Parents.

In addition to the coding changes, the patient (the proband) and his mother were heterozygous for a substitution of adenine for cysteine at position 2355, which is 375 bases (in the 3' direction) from the stop codon, on the same chromosome as the 1298C polymorphism. This substitution is in a region of unknown importance. Asterisks denote DNA-sequence variants. The open bars represent the *MTHFR* gene, and the black bars the remainder of each chromosome in the pair.

Moreover, our patient had an inborn error of metabolism in an essential precursor in a metabolic pathway, rather than an acquired deficiency, and nitrous oxide was delivered on two occasions only a few days apart.

Severe MTHFR deficiency is an autosomal recessive disorder characterized by progressive hypotonia, convulsions, and psychomotor retardation. The clinical presentation may be subtle, with the disorder manifested as developmental disability in the setting of moderate homocystinuria and hyperhomocysteinemia and low-to-normal levels of plasma methionine.²⁹ Twenty-nine mutations in MTHFR are associated with severe deficiency, with a resulting activity level that is usually 0 to 30 percent of control activity.^{7,8,14,30-33} Most patients are heterozygous for multiple MTHFR substitutions; a small minority are homozygous for mutations at this locus. The 1755G→A substitution identified in our patient occurs in a phylogenetically conserved region of the MTHFR protein (as assessed with BLASTP software, version 2.2.1). This region, which is thought to be essential for functional protein folding,³⁴ is a “hot spot” for mutations leading to MTHFR deficiency (1711C→T, 1727C→T, 1762A→T, and 1768G→A).^{7,8} The heterozygous presence of the 1755G→A substitution in the patient’s father, brother, one uncle, and one aunt and its absence in 100 independent control chromosomes suggest that it is not a benign variant.

Compound heterozygosity for the common MTHFR alleles 677C→T and 1298A→C, as seen in the patient, his mother, and his brother, causes elevations in the plasma homocysteine level⁴ that are associated with a 50 to 60 percent decrement in enzyme activity.³⁵ In the absence of coding mutations elsewhere in the MTHFR gene or evidence of a mutant splice variant, our patient’s deficient enzyme activity may be attributable to compound heterozygosity for the novel 1755G→A mutation, with the prevalent 677C→T polymorphism on the same (paternal) chromosome and the 1298A→C mutation on the maternal chromosome. It has recently been shown that when mutations causing severe MTHFR deficiency are expressed in cis configuration with the common 677C→T variant, the resultant phenotype is markedly aggravated.³⁴

Table 1. Polymorphisms in the Patient and Members of His Family.*

Family Member	MTHFR			CBS	MTR	MTRR
	677C→T	1298A→C	1755G→A	68-bp Insertion	2756A→G	66A→G
Patient	C/T	A/C	G/A	Wild type	A/A	A/G
Brother	C/T	A/C	G/A	Wild type	A/A	A/G
Mother	C/T	A/C	G/G	Wild type	A/G	A/A
Father	T/T	A/A	G/A	Wild type	A/A	A/G
Uncle 1	C/T	A/C	G/G	Wild type	A/A	A/G
Uncle 2	T/T	A/A	G/A	Wild type	A/A	A/G
Aunt 1	T/T	A/A	G/A	Wild type	A/A	A/G
Aunt 2	C/C	C/C	G/G	Wild type	A/A	A/G

* MTHFR encodes 5,10-methylenetetrahydrofolate reductase, CBS cystathionine β-synthase, MTR methionine synthase, and MTRR methionine synthase reductase.

Every year, approximately 45 million persons in North America undergo anesthesia, and nitrous oxide constitutes a major component in about half these procedures.³⁶ Because of the growing use of nitrous oxide,³⁷⁻⁴¹ patients with known mutations associated with mild or severe abnormalities in folate-cycle enzymes are increasingly likely to receive nitrous oxide. On the strength of the current findings, we believe that patients with a diagnosis of severe MTHFR deficiency should not receive nitrous oxide as anesthesia. In the case of emergency procedures, patients whose clinical presentation fits that of severe MTHFR deficiency, even if the disorder has not been diagnosed, should also not receive nitrous oxide. In the case of elective procedures, patients whose clinical presentation fits that of severe MTHFR deficiency should be evaluated, and the diagnosis should be ruled out before anesthesia with nitrous oxide is contemplated.

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