CORRESPONDENCE

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Tail Tale: $nNOS_{del1203-1434}$ Predicts Global Defects in Esophagogastrointestinal Transit

Dear Editor:

Shteyer et al reports correlative evidence of a truncating mutation of nNOSα in 2 probands (siblings) of achalasia.[1] This excellent study leaves scope for additional discussion. Achalasia results from prejunctional defects of evoked nitric oxide (NO) synthesis or postjunctional failure of signal transduction.[2] In the present study, the affected children exhibits a nonsense mutation in which amino acids beyond 1202 of $nNOS_{C\text{-}terminal}$ is deleted.[1] The authors expressed the truncated enzyme ($nNOS_{del1203-1434}$) in vitro and demonstrated absent NO production.[1] Protein gel separation analysis of the mutant nNOS shows an additional 250 kDa dimeric band, apart from the reported 135 kDa monomer.[1] Thus, dimeric nNOS may not always be surrogate for active nNOS, contrary to what has been posited.[3] Whether the ex vivo changes occur in patients is unknown as peripheral cultured fibroblasts revealed barely detectable nNOS transcripts.[1] Peripheral biomarker identification that may provide surrogate estimates of neurotransmitter contents of biopsy inaccessible tissues like the gut may address these issues. $nNOS_{del1203-1434}$ is similar in molecular mass to nNOSβ (135 kDa), which lacks the $PDZ_{N\text{-}terminal}$ and thus unable to be membrane localized for active neurotransmission. $nNOS_{del1203-1434}$ may be membrane localized due to intact N-terminal region, but still represents inactive nNOS. These aspects should be considered when examining nNOS proteome.

The motility problems were not limited to esophageal achalasia. One child had colonic perforation, which likely resulted from a distal intestinal obstruction.[1] The co-existent autism of both these children merits to be emphasized.[1] Specific genetic variants of autism are reported to result in defective enteric NO neurotransmission.[4] Whether such additional defects were present in these children may be an important area of investigation. Interestingly, like the cyclical vomiting phenotype of Phelan–McDermid syndrome,[4] both these children initially presented with refractory vomiting.[1] Manometry showed a long segment of contraction in esophageal body proximal to and including LES,[1] providing indirect evidence of functional esophageal nitrergic denervation. Repeated vomiting probably resulted from restricted gastric emptying (with achalasia also contributing), suggesting that $nNOS_{del1203-1434}$ also involved NO-neurotransmission of the pylorus. This underscores that the molecular defects in common pathways may affect both the proximal and distal gut and emphasizes the need for obtaining careful history to logically approach the possible underlying problem. Certain achalasic conditions do remain region-restricted[2] and the basis for why the features of impaired neurotransmission is not manifested throughout the entire gastrointestinal tract remains largely unknown. Despite being an orphan disease, achalasia and other motility disorders calls for investigation into enteric neurotransmission.

The authors are commended for pursuing the molecular medicine of the complex pathophysiology arising from founder mutation due to consanguinity. One child, though presented with initial failure to thrive, has now survived for 6 years,[1] probably due to residual inhibitory musculomotor neurotransmission, at least in the post-gastric regions. Because in vivo tissues were not examined for pragmatic reasons, we do not know whether there was some residual nNOS function or alternatively upregulated purinergic neurotransmission. The resected bowel specimen during ileostomy construction,[1] if available, may be examined for nNOS distribution in the nerve terminals.

The kinetics of hydride transfer during NO synthesis requires electron flow from NADPH through FAD to FMN from $nNOS_{reductase}$ to $^{heme}nNOS_{oxidase}$. The rate limiting step of electron transfer involves shift from FMN to the oxidase domain.[5] This region was unaffected in the current mutation.[1] Additionally, tail region in vitro mutants with deleted C-terminal and NADPH binding domains retain potential to synthesize NO in vitro.[6] Surprisingly, the reported genetic mutation synthesizes a mutant form with similar deletions and additional partial deletion of FAD binding region (normal $nNOS_{990\text{-}1038\ and\ 1171\text{-}1231}$), but this mutant lacks complete capacity for in vitro NO production.[1] However, $nNOS_{del1203\text{-}1434}$ demonstrated detectable NADPH oxidation,[1] suggesting misdirection of electron transfer likely to superoxide synthesis. Neither sildenafil nor Heller's myotomy provided sustained relief.[1] Exploratory and empirical pharmacotherapy to sustain the faradaic flow using $^{heme}nNOS_{oxidase}$ stimulant tetrahydrobiopterin (BH4)[7] or simply saturating the enzyme with excess calmodulin[6] to produce thermodynamically feasible electron transfer for enhancing NO synthesis may be considered. The pairing of these enzymatic augmentations with stimulus-evoked neurotransmission event is the grand challenge in designing bioelectronic medicine. Shteyer et al's study[1] emphasizes the need for examining molecular pathology in motility disorders, as impairment of nitrergic neurotransmission is not necessarily coupled with changes in nNOS expression.[8] This report[1] reiterates the need to investigate gastrointestinal motility disorders from the perspective of precision medicine.

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Reply. We appreciate the insightful comments of Dr Chaudhury and the opportunity to extend the discussion of our study. We agree that in addition to achalasia most of the phenotypes presented by these children could be attributed to the nNOS mutation. Nitric oxide is proposed to play a role in the pathogenesis of autism.[1] In Fragile X syndrome, the leading monogenic cause of intellectual disability and autism, loss of function of the RNA-binding protein FMRP[2] results in decreased nNOS translation in the developing human brain.[3] Furthermore, nNOS knockout mice exhibit altered social behavior and hyperactivity[4] with less preference for social novelty.[2] As suggested by Dr Chaudhury, our study provides insight into the pathogenesis of autism and possible therapy through restoration of NO signaling pathways.

The bowel perforation (patient II-3) could also be attributed to nNOS deficiency. $nNOS^{-/-}$ mice are susceptible to intestinal insult suggesting that nNOS serves a protective role in the intestine.[5] This may explain the spontaneous colonic perforation of patient II-3. Unfortunately, immunohistochemistry for nNOS in the intestinal specimen from patient II-3 was inconclusive due to technical problems with various nNOS antibodies. Taken together, the nNOS mutation may result in decreased ability to respond to stress, in addition to achalasia and autism. However, at this point it is unclear if other NOS isoforms (iNOS or eNOS) partially compensate for the loss in nNOS activity and lessen the severity of the observed phenotype.

We would also like to take this opportunity to comment on some of the important biochemical issues raised by Dr Chaudhury. First, caution must be used in interpreting the 135 kDa and 250 kDa bands in the SDS-PAGE of mutant nNOS. As this gel was run under denaturing conditions and was stained for protein (not immunoblotted for nNOS), the conclusion that the 250 kDa band is dimeric nNOS is not justified. However, Dr Chaudhury raises an important point that NOS dimerization should not be used as a surrogate for NOS activity. NOS dimerization is necessary for NO synthesis since electrons are shuttled from the reductase domain of one monomer to the oxidase domain of the opposite monomer during catalysis.[6] However, the heme, tetrahydrobiopterin, FMN, and FAD cofactors as well as the NADPH and arginine substrates also must bind and function properly for NO synthesis. Therefore, it is not surprising that the nNOS mutant, which is unable to properly bind FAD and NADPH, lacks NO production. Dr Chaudhury points to an active nNOS reductase domain deletion[7] of the C-terminal tail (Val-1402 to Ser-1434 in human nNOS) and an autoregulatory region in the FMN subdomain (Pro-836 to Asn-872 in human nNOS) that is not present in iNOS. However, neither of these deletions are missing the residues known to bind FMN, FAD, or NADPH and therefore cannot be directly compared to the nNOS truncation (Tyr-1202 to Ser-1434) present in these children. As pointed out by Dr Chaudhury, localization is also critical for proper nNOS function. In particular, interactions of the N-terminal nNOS PDZ domain with membrane-bound proteins are critical for proper localized NO production during neurotransmission. Conversely, the nNOS mutant observed in these children is likely localized properly but inactive.

Unfortunately, the potential therapies suggested by Dr Chaudhury (tetrahydrobiopterin or calmodulin) are unlikely improve the symptoms of these children. Tetrahydrobiopterin is critical for efficient electron transfer from and to the heme cofactor[8], but the nNOS mutant present in these children is deficient in a prior step in the NOS mechanism (hydride transfer from NADPH to FAD). Furthermore, the nNOS mutant was assayed in vitro in the presence of excess tetrahydrobiopterin and NO formation was not detectable indicating that tetrahydrobiopterin is unlikely to recover activity in vivo. Calmodulin increases the efficiency of electron transfer from FMN to the heme cofactor,[6,8] which is also not the deficient step in the nNOS mutant. In addition, calmodulin is unlikely to cross the cell membrane and calmodulin concentrations are typically not rate limiting for nNOS activity. However, Dr Chaudhury does introduce the intriguing possibility of treating these children by recovering the ability of the nNOS mutant to synthesize NO. For example, a treatment that mimicked NADPH by transferring hydride selectively to the FAD cofactor of the nNOS mutant would likely be of value in these children. Alternatively, NO donating drugs targeted to the sites where nNOS activity is deficient, may also be effective treatments.

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