When Do Gut Flora in the Newborn Produce 3-Phenylpropionic Acid? Implications for Early Diagnosis of Medium-Chain Acyl-CoA Dehydrogenase Deficiency

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Urinary excretion of 3-phenylpropionylglycine (PPG) is a diagnostic marker for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. PPG is derived from 3-phenylpropionic acid (PPA), a product of anaerobic bacterial metabolism in the gut. To determine when the infant gut was colonized with PPA-producing bacteria, we cultured stool in prereduced thiglycollate broth from 93 apparently healthy infants. We analyzed the products of bacterial metabolism by gas chromatography/mass spectrometry for the presence of PPA. Trend analysis demonstrated a significant difference (P < 0.001) in PPA production between early and later infancy. PPA was not detected in 84% of media isolated from stool collected from infants younger than four months. For older infants, 67% of the samples were PPA-positive. Thus, because the normal gut is not sufficiently colonized with PPA-producing bacteria before three to four months of age, PPG analysis alone is not a sensitive marker for the early detection of MCAD deficiency. Using stable isotope dilution mass spectrometry, we measured PPG and n-hexanoylglycine (HG) excretion in two well newborns with MCAD deficiency. HG, believed to be an endogenous metabolite associated with MCAD deficiency, was consistently above normal in all urine samples.

Additional Keyphrases: heritable disorders · phenylpropionylglycine · urine · gas chromatography/mass spectrometry · isotope dilution mass spectrometry

Medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3; MCAD) deficiency is a relatively common inborn error of mitochondrial straight-chain fatty acid oxidation in people of Northwestern European origin. The incidence in the U.K. may be 1 in 10,000 to 1 in 20,000 (1). The clinical consequences of MCAD deficiency, which vary from hypoglycemia to a Reye-like illness to sudden unexpected death (2–5), are frequently preventable when diagnosed before the onset of symptoms (2).

Although symptomatic MCAD deficiency should not present a diagnostic problem, owing to the grossly abnormal organic aciduria accompanying this disorder (6, 7), trace amounts of four specific metabolites have been implicated as useful markers in asymptomatic MCAD-deficient individuals. These markers are octanoylcarnitine, n-hexanoylglycine (HG), suberylglycine, and 3-phenylpropionylglycine (PPG) (8, 9). Unlike the other three metabolites, PPG is not a product of human fatty acid metabolism. Instead, its precursor, 3-phenylpropionic acid (PPA), is produced by anaerobic gut flora (10). Upon absorption, PPA is normally metabolized within the human mitochondria by MCAD (11). Its product after one cycle of β-oxidation is benzoic acid, which is conjugated to glycine within the mitochondria by the enzyme benzoyl-CoA: glycine N-acyltransferase (EC 2.3.1.13) (12) and excreted in the urine as hippuric acid. In the absence of MCAD, β-oxidation of PPA cannot occur. Instead, the organic acid is conjugated to glycine, presumably by the same acyltransferase, resulting in the formation of the unusual metabolite, PPG. The measurement of urinary PPG after a loading dose of PPA has been proposed as a good diagnostic test for MCAD deficiency (13) because, unlike the other metabolites, PPG appears to be unique for MCAD deficiency (14). However, studies of a few neonates with enzymatically confirmed MCAD deficiency who were not PPA-loaded suggest that PPG is not detectable in the first months of life (15, 16). One possible reason is that the gastrointestinal tract is not yet colonized with PPA-producing bacteria. The present study was undertaken to examine the evolution of gut colonization with PPA-producing bacteria.

Materials and Methods

Samples

Stool samples. Stool samples were collected from 93 apparently normal infants, ages five days postpartum to 18 months. Thirty-nine children were predominately breast-fed; 17 of these were exclusively breast-fed. Fifty-four children were formula-fed, of whom three were eating soft foods. Subjects receiving antibiotics with activity against anaerobic bacteria were excluded. To ensure that anaerobic conditions were maintained, the investigators recovered aerobic stools within 15 min of defecation, through use of the following protocol: Stool was collected only between 0800 and 1800 h, the period when nursing supervision of patients is maximal and nurses are constantly present in patients' rooms. Nursing staff notified an investigator by pager as soon as stool was discovered so that specimens were collected.

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promptly after defecation. A cotton swab was plunged into the stool to recover the maximum amount of anaerobic flora. Without delay, the specimens were placed in Port-a-Cul anaerobic transport medium (Becton-Dickinson, Cockeysville, MD). Fecal cultures were grown in prereduced thioglycollate broth at 37 °C for 48 h. The cultures were filtered through 0.22-μm (pore size) cellulose membranes before organic acid extraction.

Our protocol was based on current knowledge concerning colonic-type anaerobic organisms. Anaerobes are viable in this transport medium for as long as 72 h and the colonic flora of interest are known to be viable even after 30 min of exposure to room air (17). At 15 min of air exposure of the specimen, we demonstrated 80% to 100% viability of Clostridia, in agreement with others who have studied C. perfringens (18, 19). Preliminary evidence suggests that Clostridia species are the only producers of PPA (A. Bhala, unpublished data). We used the above procedures instead of rectal swabs out of consideration for our subjects. To our knowledge, no study has correlated the collection of stool from the ampulla to defecated stool for the recovery of anaerobic bacteria.

Urine from infants with MCAD deficiency. Urine specimens were obtained from two patients. The first was a breast-fed, full-term white female infant who was enzymatically confirmed to have MCAD deficiency (MCAD activity in cord-blood leukocytes was 0.81 nmol per minute per milligram of protein (0.31 U/g of protein); control value, 3.08 U/g). This infant has an older sibling who is also affected. The second infant was a newborn whose sibling had died after a Reye-like episode, in whom molecular and metabolite analysis confirmed MCAD deficiency; the newborn was also shown by metabolite and molecular analysis to be affected (20). Cotton balls were placed in the child's diapers and allowed to absorb urine. The cotton was placed in a plastic bottle, capped tightly, and shipped to us by overnight delivery. Samples were stored at −20 °C until they were analyzed. Both PPG and HG in standard solutions are stable for at least six months under these conditions.

Procedures

PPA extraction and analysis. Organic solvents were gas chromatography/mass spectrometry (GC/MS)-grade and obtained from Baxter Healthcare (McGaw Park, IL). Organic acids were extracted from 2 mL of salt-saturated, acidified, filtered culture medium into ethyl acetate (three times) and diethyl ether (three times). We evaporated the solvent from the pooled extracts under a gentle stream of nitrogen at 25 °C, and formed the trimethylsilyl derivatives by adding 50 μL of bis(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane (10 g/L) and 50 μL of ethyl acetate, heating at 70 °C for 30 min. The extract (1 μL) was injected onto a 25 m × 0.20 mm (i.d.) Ultra 2 capillary column fitted into a Series 5971A GC/MC (both from Hewlett-Packard, Palo Alto, CA). The initial column temperature was held at 70 °C for 5 min, after which the temperature was programmed to increase by 6 °C/min to a final temperature of 275 °C, which was held for 15 min. The injection and interface temperatures were 250 and 300 °C, respectively.

The effluent was selectively monitored for the characteristic ions of m/z 222, 207, and 104 of the monotrimethylsilyl derivative of PPA. A culture was scored as positive if all three ions were present in the ratios for authentic PPA (Figure 1) at the correct retention time (corresponding to 13.8 methylene units in our system).

PPG and HG quantification in urine. To quantify HG and PPG in the urine from the newborn with MCAD deficiency, we adapted the method of Rinaldo et al. (21). We added 29 pmol (5 μg) of n-hexanoyl-[1,2-13C]glycine and 24 pmol (5 μg) of 3-phenylpropionyl-[2-13C,16N]-glycine to 100 μL of urine. This sample was extracted into ethyl acetate and ether and the trimethylsilyl derivatives were made as described above for PPA analysis. For quantification we used the electron-impact mode, selectively monitoring the ions of m/z 230 and 232 (for HG and stable isotope-labeled HG, respectively) and m/z 351 and 353 (for PPG and stable isotope-labeled PPG). This method was sufficiently sensitive to detect concentrations of both metabolites at 50 μmol/mol of creatinine and therefore could distinguish between normal excretion [<50 μmol/mol of creatinine for each metabolite (9)] and abnormal excretion without the need for more sensitive chemical ionization detection.

Analytical recovery of HG and PPG from cotton balls. We added 10 μg of unlabeled HG and PPG in duplicate to 1 mL of non-MCAD-deficient urine, which was absorbed onto cotton balls identical to those used to collect urine. The sample was frozen to −20 °C and thawed. Liquid was squeezed out of the cotton balls and analyzed for HG and PPG contents as described above. Recovery of HG from the two samples was 92% and 95% [9.2 and 9.5 μg/mL (mg/L)] and of PPG was 95% (9.5 mg/L twice).

Statistical analysis. Trend analysis for PPA production in fecal culture media relative to infant age was
carried out by using the \( \chi^2 \)-squared test for trend (1 df). We also applied Fisher's exact test to the data.

**Results**

Figure 2 shows the distribution of PPA-positive and PPA-negative fecal cultures plotted against the infant's age at the time of stool collection. Most of the cultures in stools from infants younger than four months were PPA negative (58 of 69 samples, 84%), whereas the majority (16 of 24 samples, 67%) from infants older than this were PPA positive. Statistical analysis with the \( \chi^2 \)-squared test (1 df) for trend was significant \((P < 0.001)\). Fisher's exact test was also highly significant \((P < 0.0002)\). The whole group was a mixture of exclusively breast-fed \((n = 17)\), breast- and formula-fed \((n = 22)\), and exclusively formula-fed \((n = 54)\) infants. There were no differences in PPA production between breast-fed infants and formula-fed infants before three months of age. The number of breast-fed infants older than four months was too small to evaluate this effect.

Figure 3 shows the urinary excretion of HG and PPG from age 10 weeks to 23 weeks in a well newborn with MCAD deficiency. HG excretion was consistently high, ranging from fivefold normal (at age 16 weeks) to ~200-fold the upper limit of normal. PPG excretion, on the other hand, was not consistently increased, and in three urine samples collected at 11, 12, and 14 weeks postpartum the excretion could not easily be distinguished from normal. The sample collected at age 14 weeks had clearly normal PPG excretion.

Figure 4 shows the urinary excretion of HG and PPG from birth to day 40 in the second patient. PPG excretion was high at birth but by day 3 was clearly normal. HG excretion was consistently above normal.

**Discussion**

We investigated the production of PPA by gut bacteria in a series of healthy infants to evaluate the potential of PPG measurement for the early, presymptomatic diagnosis of MCAD deficiency. PPA-producing flora were not present in sufficient quantity to demonstrate significant amounts of PPA by selective ion monitoring mass spectrometry until ages three to four months. The majority of infants were not fully colonized until ages five to six months. The age of colonization appears unaffected by breast-feeding; however, the exclusively breast-fed sample was small, and the introduction of dietary fiber at weaning may influence the stool content and gastrointestinal flora.

Failure to detect PPA in some samples may have simply reflected failure to maintain anaerobic conditions. Examination of the sensitivities of several *Bacteroides* species, one *Peptostreptococcus* species, and two *Clostridium* species to various oxygen tensions \((17)\) indicated that they tolerated an exposure of >100 min.
with 80% viability. Given these results, our anaerobic culture techniques should ensure adequate bacterial growth of any PPA-producing organisms. In view of the closer supervision given the younger infants in our study, it is unlikely that failure to maintain anaerobic conditions occurred more frequently in neonates than in older children.

On the basis of the results of these studies, one would predict that urinary PPG excretion would be an unreliable marker for the MCAD deficiency in early infancy. Consistent with this observation, we and others have previously shown that MCAD-deficient infants do not excrete PPG in the first weeks postpartum (15, 16). However, these studies did not look at longitudinal excretion. We have now followed another two MCAD-deficient children longitudinally and have seen that, even when highly sensitive stable isotope dilution analysis is used for quantification of urinary PPG, this compound is not consistently detected in above-normal amounts. Interestingly, in the one infant from whom we were able to obtain very early urine samples, we could detect above-normal PPG concentrations until day 2, presumably arising from the infant’s inability to metabolize maternally derived PPA.

In this study, we have chosen also to follow the longitudinal excretion pattern of HG as an alternative diagnostic metabolite. In our hands and as reported by others (13, 14), this appears to be a more reliable marker for MCAD deficiency. As measured by stable isotope dilution analysis, HG excretion in this infant was consistently above normal. Therefore, we caution that PPG excretion alone should not be used as the sole diagnostic marker for MCAD deficiency in early life, and that the measurement of additional metabolites, including HG, octanoylcarnitine, and suberylglycine, should always be included in the clinical diagnostic protocol before definitive confirmation by enzyme or molecular analysis.

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References