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Stable isotopes in the diagnosis and treatment of inherited hyperammonemia

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Abstract

Stable isotopes have greatly contributed to our understanding of nitrogen metabolism and the urea cycle. The measurement of urea flux via isotopic methods has traditionally been utilized to determine total body protein synthesis in subjects with an intact urea cycle. However, isotopic studies of nitrogen metabolism are also a useful adjunct to conventional clinical investigations in the diagnosis and management of the inherited hyperammonemias. Such studies offer a safe non-invasive method of measuring the reduction of in vivo hepatic ureagenesis, and thus may provide a more accurate measure of phenotypic severity in affected patients. In addition, isotopic methods are ideally suited to evaluate the efficacy of novel therapies to augment urea production.

Keywords

Stable isotopes; urea cycle disorders; ureagenesis; mass spectrometry

1. Introduction

Tracer methodology with stable isotopes has been immensely important in numerous studies of normal and abnormal human biochemistry and metabolism. The chemical behavior of a compound labeled with stable isotopes usually is indistinguishable from that of the parent, but the presence of the label, which is readily detectable, enables research that both measures the flux through a biochemical pathway and identifies pertinent precursor-product relationships. An important advantage of using stable isotopes is that these tracers emit no radioactivity, thereby making them safe for *in vivo* studies, including pediatric investigations.

Nitrogen-15 was identified and isolated in 1937 [1], only 5 years after discovery of the urea cycle [2]. This coincidence facilitated pathbreaking research in nitrogen metabolism. Indeed, Schoenheimer, Rittenberg and other luminaries of 20th Century biochemistry exploited ^{15}N tracers to perform their seminal studies of *in vivo* protein turnover [3–6], thereby documenting the "dynamic" nature of body constituents [5,7].

Urea is the major end-product of mammalian nitrogen metabolism. The biochemical pathway required for urea synthesis consists of 6 enzymes and 2 membrane transporters [8]. Deficiencies in each of these proteins have been identified in humans, resulting in discrete

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clinical and biochemical phenotypes that have hyperammonemia as a common biochemical feature [8–11]. Severe hyperammonemia usually causes acute encephalopathy the length of which appears to correspond to the subsequent degree of learning difficulties or intellectual disability [12].

The measurement of urea production via isotopic methods is often used as an estimate of net protein catabolism in those with an intact urea cycle [3,4,13–15]. However, the application of metabolic tracers also has an important role in studying the urea cycle flux in patients with defects in the urea cycle. Such studies are not only a useful adjunct in diagnosis and management, but have also been employed to evaluate the efficacy of therapeutic interventions.

2. Measurement of isotopic enrichment

Many methods for measuring isotopic abundance are available; each has advantages and drawbacks. Initial studies employed isotope ratio mass spectrometry (IRMS), which is extremely sensitive and precise with regard to the assay of the isotopic ratio ($^{15}N/^{14}N$ or $^{13}C/^{12}C$) [16]. These instruments involve separation of gases (typically nitrogen (m/z 29/28) or carbon dioxide (m/z 45/44)) in an electromagnetic field and subsequent focusing of the labeled or unlabeled species into separate collectors, which then record the molecular abundance of each species. The ratio of labeled-to-unlabeled material then is calculated from a computerized algorithm. This technique obliges conversion of samples to a gas. It is exquisitely sensitive with respect to the measurement of isotopic abundance, but it requires that a relatively large amount of material be introduced to the mass spectrometer.

An important methodologic advance was the pairing of a gas chromatograph coupled with a mass spectrometer (GCMS), which allowed rapid separation of analytes on the GC column prior to introduction of each compound to a mass spectrometer, which assayed isotopic abundance. Most GCMS apparatuses use a quadrupole mass analyzer in order to rapidly analyze a wide array of compounds of varying molecular weight. This methodology is sensitive with respect to sample size, but it is less sensitive than IRMS for detection of isotopic abundance [17,18]. Furthermore, gas chromatography requires chemical derivatization in order to render molecules thermally volatile in the GC oven. More recently, the resolving power of GC has been coupled to IRMS. However, this necessitates an intermediate in-line combustion oven, because IRMS can only analyze simple gases. While this method obviates the need for extensive offline purification of the sample, complete separation of the analyte must occur during the chromatographic phase; whereas verification of sample purity with a quadrupole analyzer can be performed by examining its mass spectrum, when IRMS is employed it is impossible to identify the parent molecules following their combustion.

An alternative to GCMS is liquid chromatography-mass spectrometry (LCMS). The advantage of this technology is that it requires minimal sample preparation and, at least in some instances, also obviates the need for derivatization. However, with LCMS there is limited fragmentation of the parent molecule, thus restricting the ability to identify the site of isotopic labeling [16]. As with GCMS, different types of mass analyzers may be coupled with LC.

Either a GC or LC can be coupled with a tandem mass spectrometer (MS/MS), in which the fragmentation pattern of a particular molecule often allows determination of the site of the label in a large molecule [19]. However this is typically not required in the analysis of the relatively small molecules of the urea cycle. Ultimately, the choice of instrumentation depends on the analyte to be measured and the required sensitivity.

3. Stable-isotope measurement of ureagenesis in patients with inherited hyperammonemia

Stable isotope methods provide unique insight into the diagnosis and treatment of urea cycle disorders (UCDs). Such information is not accessible with traditional clinical or biochemical investigations. For instance, no standard clinical test assesses total hepatic urea production. Though this does not address any specific enzymatic defect, it accurately and non-invasively reflects the altered nitrogen metabolism, and thus may provide an objective assessment of phenotypic severity. Two studies, employing stable isotopes in different methods, not only revealed the degree of urea cycle impairment, but were sufficiently sensitive to be able to distinguish between symptomatic and asymptomatic UCD carriers [20–22].

Yudkoff and colleagues [20] evaluated a single dose of oral ^{15}N-ammonium chloride in 14 carriers of ornithine transcarbamylase (OTC) deficiency, a hemizygous OTC-deficient male who had presented with neonatal hyperammonemia and 9 control subjects. The isotopic ammonium is directly incorporated into the urea cycle via the carbamylphosphate synthetase (CPS1) reaction, thereby generating labeled urea.

In this study, the isotopic abundance of [^{15}N] urea recapitulated the clinical phenotypes, with the greatest synthesis of [^{15}N] urea observed in asymptomatic *OTC* heterozygotes and control subjects, followed by symptomatic heterozygotes and then the hemizygous male. Interestingly, despite no observable difference in urea synthesis, ^{15}N enrichment of glutamine clearly differentiated between the control subjects and asymptomatic heterozygotes, thus indicating that, despite the lack of symptoms, normal plasma ammonia, and normal urea production, nitrogen metabolism is nonetheless abnormal in OTC-deficient carriers [20]. In subsequent experiments, label incorporation into urea easily distinguished between male subjects with neonatal versus late-onset disease [21].

Lee and colleagues [22] expanded the subjects of study to also include ASS and ASL deficiency, and similarly evaluated affected patients, heterozygotes and unaffected controls. This experiment involved the constant infusion of both [^{18}O] urea and [5-^{15}N] glutamine, with subsequent measurement of both total urea and glutamine flux. Transfer of the amide-N of glutamine to the urea cycle resulted in the production of [^{15}N] urea. The [^{15}N] urea/[5-^{15}N] μglutamine ratio, a measure of urea synthesized from peripheral nitrogen sources, distinguished among unaffected control subjects, asymptomatic heterozygotes, and symptomatic individuals. Among affected individuals, this ratio was lower in those with neonatal-onset versus late-onset disease [22].

Given the current accessibility of molecular genetic testing for all of the urea cycle disorders, mutational analysis is frequently requested as part of the diagnostic work-up, especially if no pathognomonic amino acids are identified in plasma. However, pathological mutations are not always identified [23,24] and even when they are found, they may not provide much prognostic information, especially in the case of heterozygotes of OTC deficiency, the most common urea cycle disorder [25]. This may result in some scenarios where the non-invasive determination of urea cycle function can greatly contribute to clinical management.

In a notable example, Scaglia and colleagues applied the above protocol to evaluate urea cycle flux in a female with suspected partial OTCD [26] who presented at 30-months of age with developmental delay and recurrent episodes of emesis. She had hyperammonemia of 246 μmol/L (normal 22–48), and a biochemical profile suggestive of partial OTCD, including low plasma citrulline, and moderately increased urine orotic acid. Hepatic OTC activity was found to be present but reduced (770 μmol/hr/g liver; control 1500–9000 μmol/

hr/g liver). While these indices supported the diagnosis of partial OTC deficiency, molecular diagnostic testing was unable to identify a mutation. However, stable isotope studies demonstrated substantially reduced nitrogen transfer from glutamine to urea. This parameter was comparable to some affected OTCD males and even lower than that observed in some late-onset males. Ultimately, these results contributed to the risk-benefit assessment of potential avenues of long-term treatment and after consideration a decision was made to perform an orthotopic liver transplantation.

It may be that quantitation of flux with stable isotopes can not only diagnose the degree of urea cycle impairment, but even predict, prior to the onset of symptoms, whether medical management is necessary. This is particularly important for young *OTC* heterozygotes identified prospectively through affected male relatives. In fact, among 19 symptomatic and asymptomatic female *OTC* heterozygote carriers who presented with a positive family history of affected male relative, isotopic studies more accurately identified those who presented with clinical symptoms than an allopurinol challenge [27].

4. Evaluating the efficacy of therapeutics

Because isotopic studies may provide a holistic assessment of in-vivo ureagenesis, they may be used to evaluate the efficacy of novel therapies which impact urea production. Thus, urea flux may be measured before and after the intervention.

Our group has long been interested in the salutary effect of N-carbamylglutamate (NCG), a stable analog of N-acetylglutamate (NAG), the obligate activator of the CPS1 enzyme. Whereas oral NAG is hydrolyzed *in vivo*, NCG is resistant to hydrolysis. We therefore investigated the use of oral NCG as a therapy in N-acetylglutamate synthase deficiency by employing stable isotopes.

The method entails a single oral load of sodium [1-^{13}C] acetate. Oxidation of the isotopic acetate via the tricarboxylic acid (TCA) cycle results in production of CO_2 and incorporation of the label into bicarbonate. The labeled bicarbonate is then utilized by the carbamylphosphate synthetase reaction to generate ^{13}C-carbamylphosphate, the label of which is then incorporated into urea ([^{13}C] urea). As demonstrated in 17 control subjects [28], sequential blood measurements then document the appearance of serum [^{13}C] urea, whose turnover is slow compared to the period of experimental observation and provides an indicator of the production of urea over time.

We performed such investigations in a patient with NAGS deficiency, before and after a 3-day trial with NCG [28]. No changes were made in the diet or medications of this otherwise well patient. Hence, any alterations in ureagenesis were due to the action of N-carbamylglutamate. In fact, a marked increase in urea production was observed after the short trial of NCG, providing direct evidence of the salutary effect of the NCG. This was corroborated by improvements in other biomarkers, such as a decrease in plasma ammonia and glutamine, and an increase in serum urea [28].

We then employed this methodology to evaluate other conditions in which a secondary NAG deficiency is thought to reduce flux through CPS1 and result in hyperammonemia. For instance, in propionic acidemia (PA), or a congenital deficiency of propionyl-CoA carboxylase, a decrease in NAG synthesis may occur either from competitive inhibition of NAGS by propionyl-CoA [29–32] or a relative depletion of hepatic acetyl-CoA or free coenzyme A [32]. Individual case reports suggest that NCG is helpful in the treatment of PA [33–37], but these are uncontrolled studies performed during an acute illness in which the effect of NCG is difficult to differentiate from that of standard care.

We evaluated 7 subjects with PA, performing isotopic urea turnover studies before-and-after a 3-day trial of NCG. The aggregate results (Figure 1) indicate that most administered [1-^{13}C] acetate is converted to ^{13}CO2, which increases very rapidly in blood and which is unaffected by NCG treatment. NCG markedly augmented [^{13}C] urea synthesis, a phenomenon reflected by normalization of plasma ammonia levels. This not only provides direct evidence that NCG may be a useful adjunct to the treatment of hyperammonemia in PA, but additionally, offers indirect proof that insufficient activation of CPS1 may contribute to hyperammonemia in PA.

Interestingly, a marked increase in ureagenesis in response to NCG therapy was crucial in the diagnosis of a young woman with NAGS deficiency [38]. We evaluated a young patient in whom biochemical markers suggested a proximal urea cycle disorder, but in whom an allopurinol challenge was negative, and molecular diagnostic testing of *NAGS*, *CPS1* and *OTC* failed to disclose a mutation. In addition, hepatic enzyme assay showed normal activity of CPS1 and OTC. The [1-^{13}C] acetate procedure revealed a response to NCG (Figure 2) [38] which was similar to that of the NAGS deficient subject described above [28]. This made a compelling case for a diagnosis of NAGS deficiency, despite the initial negative molecular genetic testing. This prompted a search throughout the *NAGS* gene for a possible mutation, culminating in the discovery of a *NAGS* enhancer mutation. We hypothesize that this magnitude of correction to ureagenesis in response to NCG is likely unique to NAGS deficiency. Thus, this study may not only be successful in diagnosing patients in whom molecular diagnostic studies have failed, but can simultaneously validate the efficacy of the therapy.

The rationale for developing [1-^{13}C] acetate as a tracer is that, although the $^{15}NH_3$ probe enabled effective monitoring of the response to NCG [39] the taste of solutions of NH_4Cl is objectionable to most subjects and administration of ammonium salts is risky in the patient who is prone to hyperammonemia. Using [1-^{13}C] acetate as a probe neutralizes these problems, but is not without potential complications. Thus, the method depends upon rapid intestinal absorption and hepatic metabolism of the oral ^{13}C-acetate load, both before and after the NCG trial. Fortunately, it is possible to infer a problem with either absorption or oxidation of ^{13}C by careful inspection of the resulting curve of $^{13}CO_2$ formation in blood.

This experimental approach can demonstrate the efficacy of an intervention – in this instance, N-carbamylglutamate – but it does not disclose how rapidly the response to treatment has occurred. We therefore developed a protocol to address this issue. The procedure entailed a priming dose of $H^{13}CO_3$ followed by a constant infusion of this species for 5 hours. As the half-life of bicarbonate *in vivo* is short, this method permits the rapid attainment of a steady-state in bicarbonate enrichment. On the other hand, the half-life of urea is long – on the order of hours – thus, over time, as labeled bicarbonate is converted to urea, there is a constant linear increase in the blood [^{13}C] urea concentration. In our study, we introduced a single oral dose of NCG after 90 minutes of isotopic bicarbonate infusion. We then observed in 5 of 6 subjects an increase (i.e., an upward inflection) in the linear rate of appearance of [^{13}C] urea, thus demonstrating that NCG works within hours of administration.

Similar isotopic protocols may be used to evaluate novel therapies in which augmentation or restoration of ureagenesis is desired. Recent advances in gene therapy [40,41] hepatocyte transplantation [42,43] or adult hepatic stem cell [44] transplantation are examples. With each of these technologies, restoration of ureagenesis may not be uniform across the liver. Thus, liver biopsies may incorrectly determine the degree of hepatic restoration, whereas stable isotope methods presumably evaluate *in vivo* the rate of ureagenesis, and may provide a much more accurate estimate of any improvement in hepatic urea synthesis.

In conclusion, experiments utilizing stable isotopes have provided important insights into nitrogen metabolism and urea cycle disorders. Though underutilized clinically, the evaluation of *in vivo* nitrogen metabolism using tracers may provide important adjunctive information in the management of patients with urea cycle disorders. Additionally, such studies are ideally suited to evaluate the effect of novel therapies which may ameliorate nitrogen disposal via the urea cycle.

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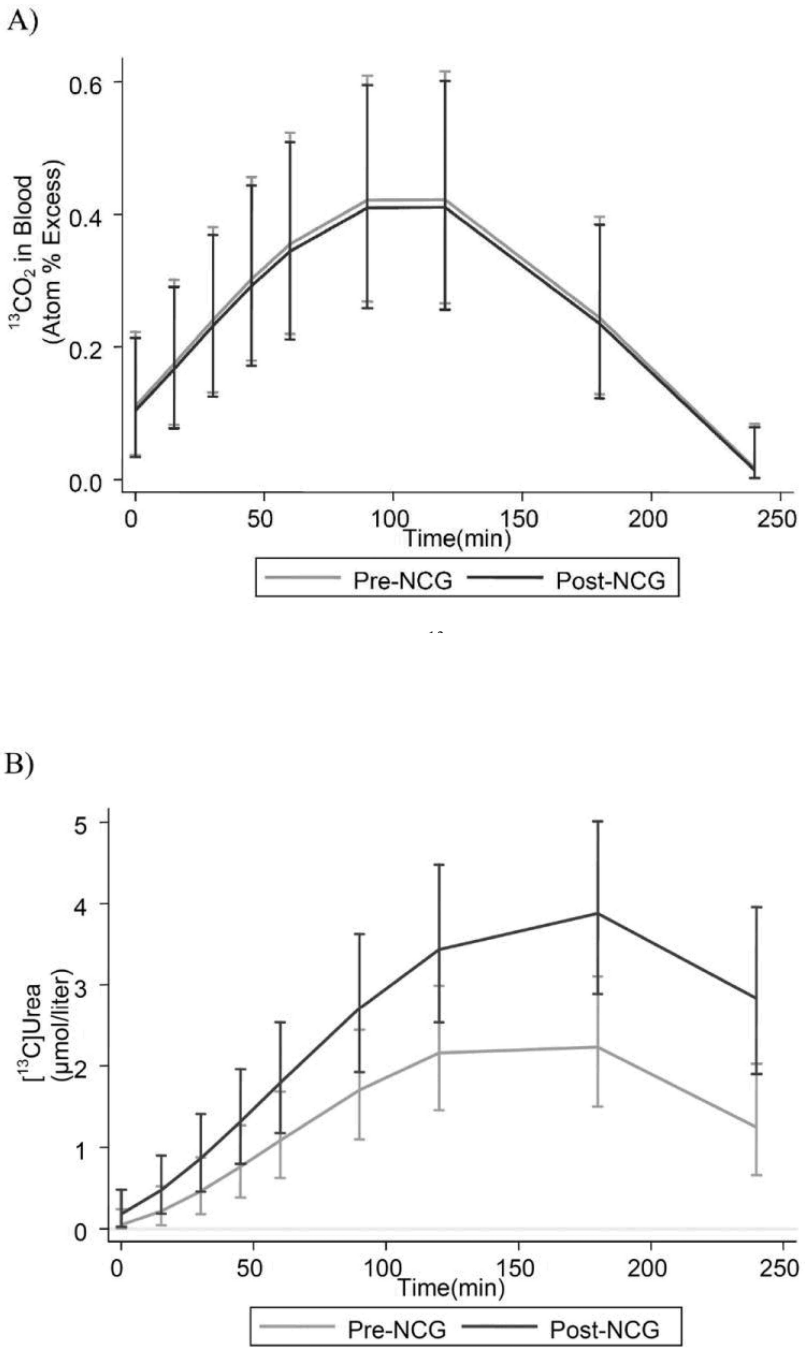

Figure 1.

Isotopic enrichment in plasma $^{13}CO_2$ (A), and plasma concentrations of [^{13}C] urea (B) in 7 patients with propionic acidemia who were administered 27.5 mg/kg of [^{13}C] sodium acetate before and after 3d NCG therapy.

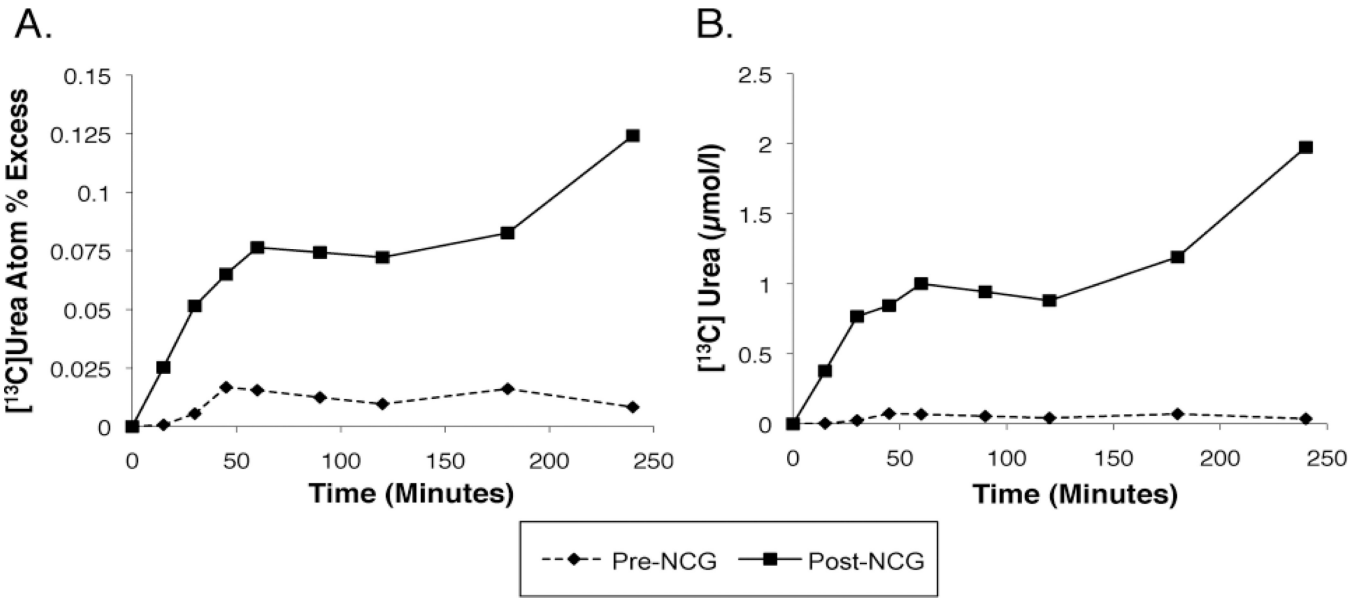

Figure 2.

Increase over time in isotopic enrichment of [^{13}C]urea (A) and plasma concentration of [^{13}C]urea (B) in a patient with a suspected proximal urea-cycle disorder, who was administered 27.5 mg/kg of [1-^{13}C]sodium acetate before and after a 3d trial of NCG. Molecular and enzymatic testing had failed to reveal a diagnosis. However, the marked augmentation in urea production following an NCG trial shown here prompted a search in the non-coding regions of the *NAGS* gene, and culminated in the identification of a mutation in the *NAGS* enhancer [38].