The effect of acute lysine administration on α-aminoadipic acid

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1.0 Background

The significance of diabetes and related co-morbidities as considerable health concerns in the US and worldwide is clearly supported by the high incidence (estimated 9.3% of the US population), mortality burden (7th leading cause of death in the US), and rising costs ($245 billion/year)[1]. Strategies to identify individuals at high diabetic risk, and to modulate disease processes in these individuals before onset of overt disease, would have significant impact in reducing mortality, morbidity, and healthcare costs. For this approach to be successful, early markers of disease that predict at-risk individuals before onset of dysregulated glycemic control are required, as well as discovering novel pathways for therapeutic targeting.

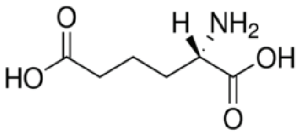

Figure 1. L-α-aminoadipic acid

The metabolite α-aminoadipic acid (2-AAA, **Figure 1**) has been established as a biomarker of diabetes risk through metabolomic profiling in epidemiological cohorts[2]. In Framingham Heart Study (FHS) participants, increased plasma 2-AAA in healthy individuals was associated with increased future risk of diabetes. In animal models, administration of 2-AAA resulted in increased insulin levels and lower fasting plasma glucose, but no change in peripheral insulin sensitivity as measured by insulin tolerance test[2]. As described in the preliminary data, *in vitro* 2-AAA treatment has been shown to enhance insulin secretion in pancreatic β cells and islets. 2-AAA is thought to have important functions in the brain[3], as well as in other organs, including liver and pancreas. Increased 2-AAA is found in skin in the setting of diabetes, as well as in chronic renal failure and sepsis, and increases with age[4]. Further, decreased urine 2-AAA has been reported in diabetes patients compared with non-diabetic[5], highlighting a likely complex feedback relationship between 2-AAA levels and early diabetes development vs. the setting of established disease.

Source of 2-AAA: 2-AAA is generated from the catabolism of the amino acid lysine through the saccharopine (mitochondrial) and/or the pipecolic acid (peroxisomal) pathways[6, 7] (**Figure 2**). Both pathways converge in the generation of 2-aminoadipate-6-semialdehyde, which is then further metabolized to 2-AAA in the cytosol[8]. 2-AAA is subsequently metabolized to 2-oxoadipate (α-ketoadipic acid), and further to acetyl-coA, entering the TCA cycle. Lysine is an essential amino acid which is acquired from dietary sources, with a recommended daily intake of 30mg/kg/day[9]. A portion of lysine in humans may be derived from the microbiome, with bacteria and fungi capable of de novo synthesis, in addition to modulation of bioavailability of dietary sources[10].

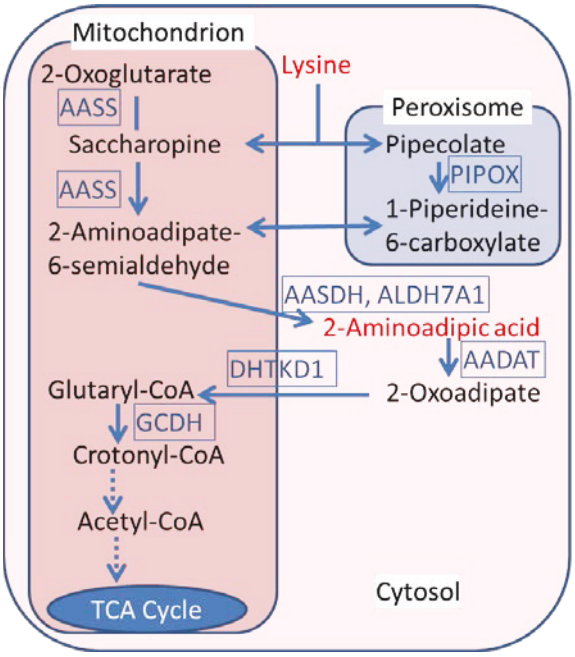

Figure 2. Multicompartmental model of L-lysine degradation (Adapted from Danhauser et al. 2012)

Genetics: Variation in genes encoding enzymes in the lysine degradation pathway (indicated in boxes in **Figure 2**) may influence 2-AAA levels and downstream function. The strongest evidence thus far exists for *DHTKD1*, variation in which has been associated with 2-Ketoadipic, 2-Aminoadipic and 2-Oxoadipic Aciduria[8, 11], and Charcot-Marie-Tooth Disease[12]. *DHTKD1* expression correlates with ATP production in mitochondria *in vitro*, and siRNA knockdown of *DHTKD1* leads to impaired mitochondrial biogenesis and increased reactive oxygen species production, resulting in apoptosis and reduced cell growth[13]. Variation in mouse *Dhtkd1* has been found to associate with expression of the gene (eQTL) and levels of protein (pQTL) in liver, as well as with serum 2-AAA levels[5]. Liver *Dhtkd1* expression correlated significantly with serum 2-AAA in these mice. Further, liver 2-AAA was negatively associated with liver mass, fasting glucose and serum cholesterol while higher liver 2-AAA was associated with higher insulin sensitivity.

2.0 Rationale and Specific Aims

Diabetes is a major global health concern, associated with significantly increased mortality and high incidence of co-morbidities. The lysine-derived metabolite α-aminoadipic acid (2-AAA) has been identified as a novel predictor of diabetes development in humans, identifying at-risk individuals before any detectable glucose abnormalities. There is exciting preliminary evidence supporting the role of 2-AAA in development of diabetes, however it is not yet clear whether 2-AAA is itself causal in diabetes development, or is a biomarker for altered metabolic processes, and many questions remain as to mechanisms of action. The aims of the study are designed to test feasibility of 2-AAA manipulation in humans through acute lysine administration.

Specific Aim: To determine whether acute lysine administration leads to increased plasma 2-AAA in humans. Catabolism of lysine leads to generation of 2-AAA. To address the hypothesis that acute lysine ingestion leads to increased circulating and excreted 2-AAA in humans *in vivo*, we will administer lysine (10g orally) to healthy volunteers (N=10), and measure the level of 2-AAA in plasma and urine at baseline and serially post-ingestion.

Sub-Study Aim: To measure clearance and excretion of lysine and 2-AAA using stable isotope tracer ^{13}C lysine (5g orally). Carbon-13 is a stable naturally occurring heavy isotope of carbon. Inclusion of a ^{13}C label on lysine allows for subsequent differentiation between endogenous and exogenous lysine and 2-AAA for calculation of clearance and excretion of the lysine bolus.

3.0 Animal Studies and Previous Human Studies

The primary goal of this study is to raise circulating 2-AAA in humans. The effects of 2-AAA administration have not been studied in humans, with no known studies of 2-AAA supplementation in humans. However 2-AAA occurs naturally in the body, and has no known adverse effects at physiological levels. Increased dietary lysine in chickens resulted in increased 2-AAA in muscle[14], however the effect of lysine on 2-AAA in humans remains to be tested in healthy subjects. Older human studies have demonstrated an increase in 2-AAA following lysine administration in children with alpha-aminoadipic aciduria[15, 16], a rare condition (< 1/1,000,000),

with variable clinical phenotypes including intellectual disability, developmental delay, epilepsy, muscular hypotonia and ataxia[8].Several studies have examined the effects of lysine supplementation, albeit without concurrent measurement of 2-AAA. Acute lysine administration in healthy subjects led to increased plasma lysine, lower plasma glucose, a small increase in insulin area, and higher glucagon. When lysine was administered in conjunction with glucose, relative to glucose alone there was a 44% reduction in the 2.5-h glucose area response, a diminished reduction in glucagon, but no change in insulin[17]. In T2DM subjects, supplementation with essential amino acids or lysine for 2 months showed some evidence of a decrease in postprandial plasma glucose[18].Other studies have shown no effect on glucose tolerance[19], fasting glucose[20], or insulin sensitivity[21]. Other lysine supplementation studies have found variable effects on other biomarkers, including plasma lipids[20], diarrhea morbidity, respiratory illness and CRP[22], anxiety, cortisol and chromogranin-A[23], and incidence of herpes simplex virus[24-26]. The stable isotope tracer ^{13}C-lysine replaces ^{12}C with ^{13}C on the lysine molecule. Previous studies have used isotope labeled lysine to calculate amino acid flux[27, 28]. Consumption of baked beef as part of a test meal led to increased plasma 2-AAA when compared with a test meal including either baked or pickled herring[29]. Treatment of overweight/obese adults with impaired fasting glucose or untreated diabetes with pioglitazone (45 mg/day) and metformin (1000 mg twice/day) led to a significant reduction in plasma 2-AAA and lysine, concurrent with an increase in insulin sensitivity[30]. Acute insulin infusion (7 hrs) in these subjects also led to a decrease in both lysine and 2-AAA[30, 31]. The epilepsy drug vigabatrin, which acts through GABA transaminase inhibition, leads to increased 2-AAA in plasma and urine, to levels similar to those seen in alpha-aminoadipic aciduria.[32]

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria:

- Men and women ages 18-45 years
- BMI 18 to <25 kg/m2

Exclusion Criteria:

- Current use of prescription medications (apart from hormonal birth control)
- Current use of amino acid supplements (including branched-chain amino acids) or supplemental protein (habitual consumption of protein powder, bars, shakes), and unwilling to temporarily discontinue use (1 week prior to study visit)
- Individuals who currently use tobacco products or have done so in the previous 30 days
- Prior or current cardiovascular disease, renal disease, or liver disease
- Diabetes mellitus (taking insulin, other anti-diabetic agents, or diet-controlled)
- Atrial fibrillation
- Bleeding disorder or anemia
- Positive pregnancy test
- Women who are breastfeeding
- Participation in another clinical trial within the previous 6 weeks prior to the study visit

- Inability to provide written informed consent
- Inability to fast for 8 hours

5.0 Enrollment

We will enroll 10 patients (N=5 male, N=5 female) who meet the inclusion and exclusion criteria. In the sub-study, we will enroll 2 patients who meet the inclusion and exclusion criteria.

The following recruitment approaches will be used: Vanderbilt CTSA (VICTR) Research Notification Distribution List, the linked email system which reaches Vanderbilt faculty and staff, as well as members of the Middle Tennessee community, and ResearchMatch, a national online registry maintained at Vanderbilt which allows people to self-register and express interest as research participants. In addition, we will contact lean subjects who had participated in our group's prior research protocols and who had expressed interest in participating in future research studies. Individuals who participate in the first study (using 10g lysine) who expressed interest in participating in future studies may be approached about participating in the sub-study for comparison purposes. Subjects may also be identified by means of prescreening the medical record, by the research coordinator.

6.0 Study Procedures

Screening

Subjects will be screened via email and/or telephone. Informed consent will be obtained via telephone with a phone consent witness. After consent has been obtained, inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Subjects who meet the inclusion criteria, and do not meet any of the exclusion criteria will be invited to participate in the study. Subjects who use protein or amino acid supplements but are willing to discontinue use for at least 1 week prior to the study may be included. Additionally, all subjects will be instructed to discontinue use of over-the-counter medications, alcohol, and strenuous exercise on the day prior to the study visit.

Subjects will be requested to record their dietary intake of all foods and beverages on the day prior to the study visit (see Diet Record Instructions, Appendix A).

Study Visit

Subjects will be requested to arrive at the Vanderbilt Clinical Research Center on the morning of the study visit (~8:00 AM) in a fasting state (at least 8 hours, with no food or drink, excluding water). After informed consent has been reviewed and documented, inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Subject's medical history and medications will be discussed and documented by study personnel to assess for any changes that may potentially exclude the subject from participating. Study

personnel will collect the subject's vital signs, height and weight prior to study interventions. All subjects will be asked to provide a urine sample for baseline 2-AAA measurement and will undergo a urine pregnancy test (if the woman is of child-bearing potential).

Following the baseline urine sample, an intravenous line will be inserted into a superficial peripheral vein to perform blood draws. Subjects will be asked to remain in a seated position for the duration of the study, but will be permitted to walk for brief periods throughout the study (e.g. to use the restroom). A baseline blood draw will be taken (time = 0 hr) for measurement of baseline plasma 2-AAA levels and related amino acids and metabolites (e.g. lysine, kynurenic acid).Immediately following the baseline blood draw, subjects will be given an oral bolus of L-lysine (10g) (Ajinomoto USA Inc, Raleigh, NC) in 100ml water. This is an amount of lysine equivalent to that which is found in a 10oz serving of beef. A similar dose of lysine has previously been shown to alter the response to glucose[17]. Blood samples will be taken at time = 30 minutes 1, 2, 3, and 4 hrs post-lysine administration. Normal (0.9%) Saline (NS) will be infused at a rate of 10 ml/hr to flush the canula prior to each blood draw, and a 5ml blood discard will be performed prior to each collection of samples. Each blood draw (including baseline) will collect about 20cc of blood, for a total collection of 120cc, or approximately 8 tablespoons of blood. Blood will be collected into one tube for serum (5ml) and 2 tubes for plasma (4ml each). Urine samples will be collected throughout the visit, in 2-hour increments (0-2 and 2-4 hrs post-lysine). Although the timing of plasma 2-AAA increases post-lysine have never been measured, peak post-prandial plasma 2-AAA levels are expected to be observed ~3hours post-lysine[29]. Plasma will be prepared from blood samples, and both plasma and urine aliquots will be frozen at -80°C and stored prior to analysis. Plasma and urine samples will be prepared for metabolite measurement by methanol extraction and centrifugation in Dr. Ferguson's laboratory using standard protocols[33]. 2-AAA and other metabolites and biomarkers (e.g. lysine, creatinine) will be measured at Vanderbilt core facilities (e.g. Mass Spectrometry core).

Compensation

Subjects will receive $150 following successful completion of the study visit.

Biomarkers

Blood and urine samples will be coded for subject confidentiality. Measurements of interest will be performed at Vanderbilt University. For possible future investigations, excess blood and urine samples will be frozen and stored in Dr. Ferguson's laboratory.

6.1 Sub-Study Procedures – Lysine Tracer

Screening

Of subjects who successfully completed the primary study, we will invite two individuals to return for a second visit. If we are unable to recruit from our original enrollees, we will recruit from within the original pool of interested volunteers or from participants who expressed interest in our future studies, using the same screening procedures described above. Subjects will be

asked about any changes in their health and medications that have occurred since their participation in the lysine study.

Study Visit

Subjects will be requested to arrive at the Vanderbilt Clinical Research Center on the morning of the sub-study visit (~8:00 AM) in a fasting state (at least 8 hours, with no food or drink, excluding water). After informed consent has been reviewed and documented, inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Subject's medical history and medications will be discussed and documented by study personnel to assess for any changes that may potentially exclude the subject from participating. Study personnel will collect the subject's vital signs, height and weight prior to study interventions. All subjects will be asked to provide a urine sample for baseline 2-AAA measurement and will undergo a urine pregnancy test (if the woman is of child-bearing potential).

Following the baseline urine sample, an intravenous line will be inserted into a superficial peripheral vein to perform blood draws. Subjects will be asked to remain in a seated position for the duration of the study, but will be permitted to walk for brief periods throughout the study (e.g. to use the restroom). A baseline blood draw will be taken (time = 0 hr) for measurement of baseline plasma 2-AAA levels and related amino acids and metabolites (e.g. lysine, kynurenic acid).Immediately following the baseline blood draw, subjects will be given an oral bolus of ^{13}C L-lysine (5g) (Cambridge Isotope Laboratories, Tewksbury, MA) in 50ml water. This is an amount of lysine equivalent to that which is found in a 5oz serving of beef. Blood samples will be taken at time = 30 minutes 1, 2, 3, 4, 5 and 6 hrs post-lysine administration. Normal (0.9%) Saline (NS) will be infused at a rate of approximately 10 ml/hr to flush the canula prior to each blood draw, and a 5ml blood discard will be performed prior to each collection of samples. Each blood draw (including baseline) will collect about 20cc of blood, for a total collection of about 160cc, or approximately 11 tablespoons of blood. After a 3-5ml discard, blood will be collected into one tube for serum (5ml) and 2 tubes for plasma (4ml each). Urine samples will be collected throughout the visit, in 2-hour increments (0-2, 2-4, and 4-6 hrs post-lysine). Plasma will be prepared from blood samples, and both plasma and urine aliquots will be frozen at -80°C and stored prior to analysis. Plasma and urine samples will be prepared for metabolite measurement by methanol extraction and centrifugation in Dr. Ferguson's laboratory using standard protocols[33]. 2-AAA and other metabolites and biomarkers (e.g. lysine, creatinine) will be measured at Vanderbilt core facilities (e.g. Mass Spectrometry core).

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Biomarkers

Blood and urine samples will be coded for subject confidentiality. Measurements of interest will be performed at Vanderbilt University. For possible future investigations, excess blood and urine samples will be frozen and stored in Dr. Ferguson's laboratory.

7.0 Risks of Investigational Agents

Lysine Administration: Lysine is expected to be well-tolerated by all participants. Lysine is used as a dietary supplement, available over the counter, with no known long-term risks. There are no additional risks associated with the use of stable isotope tracer ^{13}C lysine. The proposed dose of lysine may cause mild gastrointestinal upset in some subjects. This is expected to be minor and transient.

Venous Blood draw: This is a routine procedure that is considered standard of care in clinical medicine. At the study visit, subjects will undergo venous blood draws. All blood draws will be performed by trained personnel using universal precautions to protect both the subject and personnel. The risks to subjects are minimal, but may include pain, allergic reaction, infection or bleeding at the needle stick site. These usually resolve without any specific medical therapy over the course of minutes to days.

Private Health Information: This information will be collected during the course of the study. However, only key study personnel will have access to this information, which will be stored in a HIPAA compliant, password protected REDCap database. No protected health information will be shared with employers, insurers, or non-research personnel

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Adverse events will be reported to the IRB per Vanderbilt University IRB policy.

9.0 Study Withdrawal/Discontinuation

Subjects may withdraw from the study at any time and should notify study personnel if they wish to withdraw from the study. Subjects may request their biological samples to be destroyed at any time. However, any data or biological samples that have already been used for research cannot be destroyed. Subjects may be discontinued from the study at the discretion of the investigator (possible reasons listed below). Subjects will receive financial compensation for completion of the visit.

Possible reasons for withdrawal/discontinuation from study include, but are not limited to:

- Noncompliance with treatment or procedures
- Decision by participant/participant withdraws consent
- Significant adverse event deemed by investigator to preclude continued participation

10.0 Statistical Considerations

Given the pilot nature of this proposal, the primary goal is to detect whether there is a measureable increase in 2-AAA in plasma or urine following lysine administration.

Based on preliminary analyses in a separate study (N=120 healthy individuals), we expect a mean baseline 2-AAA of 0.00299 with a standard deviation of 0.0025 (arbitrary units based on mass spec measurements). Accounting for this between-subject variability, in order to detect a significant change in 2-AAA post-lysine, we will require a mean post-lysine 2-AAA concentration of 0.0055 (at 80% power, α=0.05). We expect the actual increase to be larger than this, and thus expect to be well-powered to detect a difference in this pilot sample. Although no data exist for the expected change in 2-AAA, a dietary study measuring 2-AAA following protein-rich meals reported a considerable post-prandial increase in 2-AAA (e.g. > 10 fold increase)[29].

The change in 2-AAA will be analyzed both as the overall area under the curve, and as the change from baseline to each subsequent timepoint (analyzed by paired T tests).

11.0 Privacy/Confidentiality Issues

Strict confidentiality will be maintained to the fullest extent by the research team, including keeping all data in a secure location. All specimens will be coded after they are obtained and the code key kept in a secure location. Blood samples will be coded anonymously to remain confidential and identifiers will be kept in a separate, secure location. Samples may be shared with third parties outside of Vanderbilt for future testing but will remain anonymous to the recipient. Subjects may contact the principal investigator at any time to request that samples be destroyed.

12.0 Follow-up and Record Retention

Anticipated study duration is 1-2 years. Research data will be maintained by the PI after study closure. After study closure, research data will be maintained for a minimum of 6 years and possibly indefinitely. Data will be stored on the Vanderbilt University computer network in a password-protected database. Only members of the study team will have access. Pertinent paper documentation will be kept in a locked office and only study personnel will have access. Only personnel directly involved with the study will have access to source data and the electronic database.

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Appendix A

Diet Record Instructions

You will need to write down ALL food and beverages consumed on the assigned day. Please take the diet record with you all day during the day you are recording intake and record items at the same time you consume any food or beverage. There are four columns that need to include information about a food or beverage you consume: Time, Food/beverage, Amount, and Description/Preparation.

Time: List the time you consumed each food or beverage

Food/Beverage: Write down the name of the food or beverage. Use brand names (e.g. Burger King, Big Mac, Lean Cuisine) whenever possible. Remember to list all additions to foods and beverages such as cream, sugar, butter, jelly, lemon, ketchup, etc. For mixed dishes (e.g. pizza, casseroles, etc) list each food item and try to include the amount for each item. If you use a recipe, divide the total amount used by the number of servings you had. For example, if you used .5 lb of 85% lean ground beef to make a meat loaf that made 4 servings and you had 1 serving, the total amount of group beef you should include is 1/8 of a pound. Or if possible, you may provide the recipe and list what proportion of the entire recipe you ate.

Amount: List the amount eaten or drank in ounces (oz), teaspoons (tsp), tablespoons (tbsp), cups (c) or in units such as 1 slice of whole wheat bread, or 1 orange. When listing meats, list for cooked amounts.

Description/Preparation: Describe methods used to prepare food or beverages (ex: baked, grilled). Include any oil or other products used in the preparing of food (e.g. 1 tsp olive oil to sauté vegetables). Include brand names or fast-food and restaurant names when possible

Reminders List: Please use this list to remind you to include all food and drink that you consume.

1. Record all snacks you ate during each day.

2. Record all beverages such as coffee, tea, water, milk, soda pop, juice, alcohol, etc.

3. Include any items you added to your food or drink such as cream, milk, sugar, lettuce, tomato, ketchup, mustard, pickles, butter, margarine, etc.

4. Record the time of the day when you eat any food or beverage

5. Make sure to describe how each food was prepared (baked, fried, broiled, etc)

BE AS DESCRIPTIVE AS POSSIBLE

The following is an example of a diet record. Please note how detailed it is.

Time	Food/Beverage	Amount	Description
8:00 AM	cereal	1 cup	Cheerios
	milk	½ cup	2%
	bread	2 slices	whole wheat
	jelly	1 tbsp	Smuckers, strawberry
	juice	6 oz	Tropica, orange, no pulp
12:00 PM	pizza	2 slices	16" pizza from Pizza Hut, thin crust with mozzarella cheese, tomato sauce, and pepperoni
	Coke	12 oz	
	Cookies	3	chocolate chip, medium size
7:00 PM	Chicken	3 oz	baked, skinless, boneless
	olive oil	1 tbsp	used to cook chicken
	lemon juice	1 tbsp	drizzled over chicken
	salad	1 cup	ingredients listed below
	lettuce	4 leaves	iceberg
	red tomato	¼ of tomato	raw
	cucumber	4 slices	peeled
	croutons	6 each	onion flavored, Pepperidge Farms
	Dressing	2 tbsp	Low-fat ranch, Ken's
	French fries	¾ cup	crispy, baked,
10:00 PM	apple	1 each	red and delicious, medium
	coffee	6 oz	milk added (see below)
	milk	1 tbsp	2%

Name: _____ Day: _____

Date: _____

DIET RECORD

Time	Food/Beverage	Amount	Description