Amino acids stimulate leg muscle protein synthesis in peripheral arterial disease

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Objective: Older patients with peripheral arterial disease (PAD) and intermittent claudication have impaired walking ability resulting from reduced lower extremity blood flow. Evidence suggests that leg muscle abnormalities may also contribute to walking intolerance in claudicants. In healthy elderly people, leg muscle protein synthesis can be augmented by nutritional supplementation with amino acids; preliminary data suggest that this increases muscle mass, walking ability, and functional status. In this study, we investigated whether amino acid supplementation would improve leg muscle protein synthesis in elderly PAD subjects, given that reduced leg blood flow might restrict the availability of amino acids to muscle.

Methods: Two groups participated in the study: a group of 11 claudicants (mean age, 62 years; mean ankle-brachial index, 0.62; 46% male) and a group of 9 age- and sex-matched healthy controls (mean ankle-brachial index, 1.1). Both groups underwent measurement of leg blood flow by using strain gauge plethysmography, as well as measurement of baseline and amino acid–stimulated protein synthesis in leg muscle. Protein synthesis was quantified from calf muscle biopsy samples by measurement of the fractional synthetic rate (FSR) of protein, by using the incorporation of the stable isotope L-[ring-2H5]phenylalanine into muscle protein. Total protein was extracted from muscle samples, and gas chromatography/mass spectroscopy methodology was used to measure incorporation rates. After measurement of basal FSR, all subjects were given an oral drink of 15 g of essential amino acids, and the measurements of FSR were repeated. Data are expressed as mean ± SD; statistical analysis of differences between the two groups (with and without amino acid supplementation) was performed by using analysis of variance with repeated measures.

Results: Calf blood flow was reduced in the PAD subjects compared with controls (1.44 ± 0.53 mL/min per 100 mg of tissue vs 2.40 ± 0.57 mL/min per 100 mg of tissue; P = .005; t test). FSR in the basal state was equivalent between the two groups (healthy, 0.060% ± 0.025% per hour; PAD, 0.061% ± 0.029% per hour; P = .97). Equivalent increases (P < .05) occurred in both groups in response to oral amino acid supplementation (healthy, 0.087% ± 0.012% per hour; PAD, 0.104% ± 0.041% per hour; P > .05; analysis of variance).

Conclusions: Despite reduced leg blood flow, elderly PAD patients synthesize calf muscle protein in the basal state in a fashion similar to that in healthy elderly people. More importantly, administration of exogenous amino acids produces a significant increase in protein synthesis in these patients that is also equivalent to that in healthy elderly people. Our goal is to use these results as the basis for an intervention study to determine whether long-term oral amino acids, by augmenting calf muscle protein synthesis, increase calf muscle mass, walking ability, and functional status in elderly claudicants. (J Vasc Surg 2007;45:554-60.)
kludicants is decreased compared with that of healthy elderly people and that stimulation of protein synthesis by exogenous AAs is also impaired.

METHODS

Subjects

Eleven older claudicants and nine healthy elderly volunteers participated in this study. Claudicants were recruited from the Vascular Surgery Clinics at the University of Texas Medical Branch (UTMB) and were identified by typical symptoms of lower extremity muscle pain brought on by walking and relieved by rest, together with resting Doppler-derived ankle-brachial indices (ABI) of 0.9 or lower. Healthy control subjects were recruited from the Scaly Center for Aging volunteer registry at UTMB. Exclusion criteria included current participation in a muscle-strengthening program, currently on a weight-loss diet, recent (within 6 months) ingestion of anabolic steroids or corticosteroids, or recent (within 6 months) lower extremity revascularization. All subjects were fully informed about the purpose and procedures of the study and provided written consent according to the guidelines established by the Institutional Review Board at UTMB, which approved the protocol before initiation of the study.

Experimental protocol

Overview. Calf (gastrocnemius) muscle protein synthesis was quantified by measuring the incorporation of a stable isotope of the essential AA (EAA) phenylalanine into the muscle protein by using gas chromatography/mass spectrometry methodology.6-10 Muscle samples were obtained by biopsy. To ensure that a steady state with respect to the enrichment of the stable isotope was maintained, enrichments were measured on blood samples obtained from an arterialized hand vein throughout the protocol.11,12 Protein synthesis was measured in the basal, postabsorptive state and after administration of an oral bolus of EAs.

Protocol. The experimental protocol (Fig 1) was performed in the General Clinical Research Center at UTMB. All study participants were instructed to maintain their normal diet in the weeks preceding the study. Participants were admitted to the General Clinical Research Center the night before each study, underwent an overnight fast, and were allowed only water on the day of the study.

On the morning of the study, polyethylene catheters were inserted into a forearm vein for infusion of L-[ring-2H5]-phenylalanine (Cambridge Isotope, Andover, Mass) and into the contralateral upper extremity dorsal hand vein for arterialized blood sampling. A blood sample was obtained for measurement of the background AA enrichment. A primed continuous infusion (priming dose, 0.05 mol·kg·min⁻¹) of labeled phenylalanine was then initiated through the forearm vein catheter and continued throughout the study. Before infusion, it was dissolved in 0.9% sterile saline and filtered through a 2-μm filter. Venous blood collected from the dorsal hand vein was “arterialized” by wrapping the hand in a heating blanket.11,12 Blood samples were collected from this catheter hourly throughout the study to establish the presence of an arterial steady state of phenylalanine enrichment.

Arterialization of the venous blood was confirmed by hourly blood gas analysis.12 Muscle biopsies (50 mg) were obtained from the gastrocnemius muscle of one leg of each participant under sterile conditions and local anesthesia (1% lidocaine with added 8.4% sodium bicarbonate mixed 1:10) by using a 5-mm Bergstrom biopsy needle. After administration of the local anesthetic, an incision (1-2 mm) through the skin and superficial fascia was created; all biopsy samples were obtained from the same incision site, but at 180° to each other and 2 to 3 mm apart to minimize inflammatory interference. Biopsies were performed at 120, 240, and 480 minutes after initiation of the stable isotope infusion (Fig 1). After each biopsy, muscle samples were rinsed with sterile saline, blotted dry, immediately frozen in liquid nitrogen, and stored at −80°C for later analysis.

The basal, postabsorptive rate of gastrocnemius muscle protein synthesis was measured by using the first two muscle biopsy samples by quantification of the fractional synthesis rate (see below). After the second biopsy, each participant ingested an oral mixture of 15 g of EAs (Table I) dissolved in a noncaffeinated, noncaloric soft drink. The composition of the EAA drink was developed to approximate the distribution of EAA in skeletal muscle and the amount required to produce an increase in the intracellular concentrations of the EAs in relation to their respective contributions to muscle protein synthesis.9,13,14 The underlying assumption in this stable isotope methodology is that an isotopic steady state is achieved and maintained.15 Therefore, on the basis of previous studies, it was determined that while using a constant infusion of L-[ring-2H5]-phenylalanine (0.05 mol·kg⁻¹·min⁻¹), an additional 0.186 g of L-[ring-2H5]-phenylalanine should be added to the EAA drink to maintain the isotopic enrichment (tracer/tracee ratio) in the blood at approximately 0.08 (isotopic steady state).9,13,14 The EAA-stimulated rate of muscle protein synthesis was calculated by using the time interval between the second and third muscle biopsies (Fig 1).

After the final muscle biopsy, calf blood flow was measured by using strain gauge plethysmography in accordance...
with previously described methods.\textsuperscript{16,17} Briefly, the plethysmographic examination was performed at room temperature and was performed on both legs simultaneously with the subject in the supine position. Mercury-in-silastic strain gauges (Hokanson, Bellevue, Wash) were placed around the calf muscle at the point of greatest circumference,\textsuperscript{17} and cuff inflators were used to rapidly inflate and deflate blood pressure cuffs placed around the thighs and ankles bilaterally. The blood flow output signals were transmitted to a recorder. Calf blood flow values were expressed as milliliters per minute per 100 mL of leg tissue volume. At the end of the protocol, peripheral lines were removed; subjects were provided with a meal and were monitored for 1 to 2 hours before discharge.

**Analytical methods**

**Blood.** Arterialized blood samples from the retrograde catheter in the dorsal hand vein were collected for PO\(_2\) measurement and serum AA enrichment. PO\(_2\) was measured by a Rapidpoint 405 System (Bayer HealthCare, Clayton, NC) in the Department of Pathology, UTMB. Blood for AA enrichment was immediately mixed in preweighed tubes containing 15\% sulfosalicylic acid solution. Samples were reweighed and centrifuged, and the subsequent supernatant was removed and frozen at \(-80^\circ\text{C}.\) Upon thawing, blood AAs were extracted from 500 \(\mu\)L of supernatant by cation exchange chromatography (Dowex AG 50W-8X; 100-200 mesh H\(^+\) form; Bio-Rad Laboratories, Richmond, Calif) and dried under a vacuum (Savant Instruments, Farmingdale, NY). Phenylalanine enrichments and concentrations were determined on the tert-butyldimethylsilyl derivative by using gas chromatography/mass spectrometry (GC-MS HP model 5989; Hewlett-Packard, Palo Alto, Calif) with electron impact ionization. Ions 336 and 341 were monitored.\textsuperscript{18}

**Muscle.** Twenty to twenty-five milligrams of muscle was homogenized in 800 \(\mu\)L of 10\% perchloric acid. The free intracellular enrichment of phenylalanine was measured on the supernatant obtained after tissue homogenization and centrifugation. The bound phenylalanine (that incorporated into muscle protein) was measured from the pellet obtained after centrifugation. The pellet was washed and dried, and the proteins were hydrolyzed in 6 N HCl at 110\(^\circ\text{C}\) for 24 hours. The hydrolysate was processed in the same fashion as the blood samples, and the phenylalanine enrichment was measured by gas chromatography/mass spectrometry by using chemical ionization as described by Calder et al.\textsuperscript{18}

**Protein synthesis calculation**

Gastrocnemius protein protein synthesis was quantified by calculation of the fractional synthetic rate (FSR) of total muscle protein, by measuring the incorporation rate of the stable isotope of phenylalanine (\(L-[\text{ring-2H}_5]\)-phenylalanine) into muscle protein. Muscle FSR was calculated by dividing the increment in enrichment in the protein-bound phenylalanine tracer/tracee ratio by the enrichment in the free intracellular phenylalanine tracer/tracee ratio in the basal period and after oral EAA administration.\textsuperscript{6,8} (Fig 2).

**Sample size calculation**

The sample size was calculated by anticipating a change of 50\% or greater in all measurements, on the basis of previous studies performed by our group.\textsuperscript{6-10} These studies demonstrated coefficients of variation for changes in various parameters of protein synthesis in the range of 15\% to 30\%. Therefore, the sample size was calculated on the basis of the number of subjects required to demonstrate a difference in a parameter with a coefficient of variation of 30\%. On the basis of the assumption of a type I error of 0.05 and a power of \(\beta\) of 0.8, we calculated that eight subjects per group should be sufficient to detect a 50\% difference.

**Statistical analysis**

Differences between the two study groups were analyzed by using the \(t\) test assuming equal variances and \(\chi^2\) analysis. Comparison of the rates of protein synthesis between the two groups before and after EAA ingestion were performed by using analysis of variance with repeated measures. Differences were considered significant if \(P < .05\).
RESULTS

PAD and control groups were matched with respect to age and sex (Table II). Hypertension, diabetes mellitus, dyslipidemia, coronary artery disease, and smoking (ever) were more prevalent in the claudicant group (Table II). Calf blood flow and ABI were significantly reduced in the claudicant group compared with the control group (Table III).

A steady state with regard to phenylalanine enrichment was achieved within 60 minutes of initiation of the labeled phenylalanine infusion and was maintained throughout the study period. Confirmation of arterialization of the venous blood was obtained by measurement of PO₂; the mean PO₂ was 59 ± 6 mm Hg (Fig 3).

FSR in the basal state was not significantly different between groups (healthy, 0.060% ± 0.025% per hour; PAD, 0.061% ± 0.029% per hour; \( P = .97 \)). Significant increases (\( P < .05 \)) occurred in both groups in response to oral AA supplementation (healthy, 0.087% ± 0.012% per hour; PAD, 0.104% ± 0.041% per hour); the increases between the two groups were equivalent (\( P > .05 \); analysis of variance; Fig 4).

DISCUSSION

The most important finding of this study was that despite reduced calf blood flow, older claudicants synthesize calf muscle protein in a fashion similar to that in healthy elderly people and are able to respond to a single oral bolus of EAAs in a similar manner. The next step will be to determine whether longer-term AA administration will improve leg muscle mass and strength in claudicants and whether this in turn will improve walking ability and functional status. Preliminary data obtained by our group suggest that this is possible for healthy elderly people. Sixteen weeks of oral AA supplementation (15 g of EAAs administered twice a day) to these individuals resulted in increased leg muscle mass, quantified by magnetic resonance imaging, and leg strength, quantified as increased one-repetition maximum, as well as improvements in functional status measured by summary performance scores (unpublished data).

Previous studies addressing nutritional supplements in the elderly have produced mixed results. Fiatarone et al administered multinutrient supplements to 100 frail nursing home residents over a 10 week period and found no effects on muscle strength, thigh muscle cross-sectional area, gait velocity, or stair-climbing ability. Milne et al performed a meta-analysis of 55 randomized controlled trials (9187 subjects) addressing oral protein and energy supplementation in the elderly and found that supplements decreased mortality and complication rates for undernourished hospitalized elderly patients but did not seem to have any benefits on functional status or in well-nourished subjects. The differences between these findings and ours may be related to the amount and composition of the protein in...
the supplements, as well as the inclusion of other nutrients, such as carbohydrates. Evidence exists that the inclusion of carbohydrates in nutritional supplements for the elderly is not beneficial and may actually reduce the benefits of AAs alone. Additionally, previous studies have shown that elderly subjects who take supplements reduce their normal dietary intake correspondingly, meaning that they do not experience increased total daily energy intake. Hence, to be effective, a nutritional supplement for the elderly should stimulate muscle anabolism more efficiently than regular food, so that if normal intake is reduced, total energy intake is still increased. In regard to this, we have found that the improvements elderly subjects experience from AA supplements are associated with EAAs; non-EAAs are truly non-essential in terms of beneficial effects on muscle. Traditional protein supplements contain a large proportion of non-EAAs, and this may also detract from their overall benefit.

Studies of nutritional supplements in patients with PAD have been very limited and have also produced mixed results. A meta-analysis of omega-3 fatty acid administration in claudicants demonstrated no benefits on walking distances or ABIs. A pilot study demonstrated a trend toward an increase in walking distance with L-arginine (the precursor of nitric oxide and a stimulator of endothelium-dependent vasodilation) in 20 claudicants. A carbohydrate supplement increased walking times in a small group of claudicants already participating in an exercise rehabilitation program. This paucity of data provides further support for additional trials of nutritional supplements in the treatment of PAD.

Originally we hypothesized that the reduced blood flow found in patients with IC might restrict the delivery of AAs to calf muscle and that this would reduce both basal and AA-stimulated calf muscle protein synthesis. A previous study performed by our group demonstrated that muscle protein synthesis in healthy subjects was regulated by the concentration of AAs in blood rather than by the intracellular concentration. In another study, changes in leg muscle protein synthesis were directly and significantly correlated with changes in leg blood flow in healthy young subjects treated with insulin. However, Short et al found that although administration of prednisone decreased leg blood flow by 25% in healthy young people, there was no effect on leg muscle protein synthesis. We speculate that although increasing leg blood flow may increase protein synthesis, the reduction in flow observed in patients with claudication (40% in this study) is not of sufficient magnitude to restrict the availability of AAs to calf muscle to a degree necessary to reduce protein synthesis. Indeed, preliminary data obtained by our group in a rabbit model showed that reduction of leg blood flow by one third by using angioplasty balloons had no effect on either basal or AA-stimulated leg muscle protein synthesis (unpublished data).

These findings have significant implications for the treatment of IC. At this time, the options for noninvasive treatment are limited. Exercise rehabilitation, although effective, is not applicable to many patients who cannot exercise sufficiently to improve walking ability because of comorbidities such as coronary artery disease and osteoarthritis. Studies have shown that home exercise programs are much less effective than supervised programs, yet many patients are unwilling or unable to travel to centers for supervised exercise several times per week. Only one medication, cilostazol, is currently approved by the US Food and Drug Administration for the treatment of claudication. Side effects, cost, and contraindications prevent its use in many patients. Invasive procedures such as angioplasty and stenting have produced mixed results regarding improvements in functional capacity and quality of life in patients with IC, and surgical bypass is unsafe in many patients. There is a need for more widely applicable, effective treatments to improve functional status and quality of life in patients with this common disease. It is our hope that AA supplementation may ultimately prove to be one of these treatments.

**AUTHOR CONTRIBUTIONS**

Conception and design: LAK, DT, RRW
Analysis and interpretation: LAK, DT, GCH, RRW
Data collection: LAK, DT, JB
Writing the article: LAK, DT, JB
Critical revision of the article: LAK, DT, JB, GCH, RRW
Final approval of the article: LAK, DT, JB, GCH, RRW
Statistical analysis: LAK
Obtained funding: LAK
Overall responsibility: LAK

**REFERENCES**


Submitted Sep 14, 2006; accepted Nov 10, 2006.

DISSCUSSION

Dr Ronald Webb (Oakland, Calif): First of all, I would like to thank the program committee for asking me to review this paper. It forced me to review a little bit of biochemistry. Second of all, I would like to thank Drs Dilley and DeLaria for having this meeting at what I think has been an exceptionally nice venue, and I would also like to thank Dr Killichew for communicating with me by e-mail with this paper several weeks ago. It is also interesting this is the first day in Washington for peripheral arterial disease to discuss a paper with regards to amino acids and peripheral arterial disease. There are really three things that have had level I evidence that improved claudication distance, and one has been smoking cessation, one has been supervised exercise, and the third has been Pletal.

There are other drugs that we are using now in the office that I think help us with the management of the claudicant. The statin drugs of interest have been shown to actually lower the C-reactive protein which you know is an inflammatory index of carotid artery disease. Plavix has improved the claudication distance with which I think has been an exceptionally nice venue, and I would like to thank Drs Dilley and DeLaria for having this meeting at what I think has been an exceptionally nice venue, and I would also like to thank Dr Killichew for communicating with me by e-mail with this paper several weeks ago. It is also interesting this is the first day in Washington for peripheral arterial disease to discuss a paper with regards to amino acids and peripheral arterial disease. There are really three things that have had level I evidence that improved claudication distance, and one has been smoking cessation, one has been supervised exercise, and the third has been Pletal.

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This paper does seem to show that nutritional supplementation can increase the muscle mass of claudicants. However, this paper does not document that the pain of claudication or the distance of claudication improved with nutritional supplementation. Will Hiatt at the University of Colorado has shown an increase in treadmill distance after giving 2 grams of carnitine twice daily orally.

My questions to the author are, one, what mixture of essential acids and nonessential amino acids do you feel could improve the exercise tolerance of a claudicant? Number two, why did you choose phenylalanine rather than leucine, arginine, or carnitine for this study? And what type of study would you design to prove you could increase claudication distance—a 6-minute walk test, gaited treadmill exercise, peak blood flow with plethysmography, or Doppler indices?

I appreciate having the opportunity to discuss this paper.

Dr Louis Killewich: Thank you very much, Dr Webb, for your comments. I guess I have an advantage here; the questions are right here up where I can see them. What mixture of essential and nonessential amino acids do you feel could improve the exercise tolerance of claudicants? Well interestingly I did not really go over it in my paper, but the work on essential amino acids was started by a large group of physiologists in Galveston headed by Dr Wolfe, whom you alluded to in your comments. They have studied in the past essential versus nonessential amino acids, and they found that the nonessential amino acids, at least in healthy elderly, had no benefit. That is why in my study I have used essential amino acids only and the studies we plan to do in the future will be using only essential amino acids.

Why we chose phenylalanine rather than a different stable isotope basically has to do with the cost, the availability, and the fact that, for example, the lucine stable isotope is oxidized in muscle, and so if you are going to use that as your tracer for protein synthesis, you have to do a lot of additional calculations and measurements in order to account for the oxidation. It is really just an ease of use of the isotope.

And what type of study would I design to prove that I could increase claudication distance? Well, the study that we are starting now, we are going to give amino acid supplements to claudicants for a 16-week period and our end points are going to include both treadmill testing and the 6-minute walk test. The 6-minute walk test is a very simple test of claudication, which actually was developed by Andy (Gardner), and it involves having a patient walk in a hallway between cones. There is no treadmill, no graded treadmill, or constant load treadmill, and it has been validated in the sense that it is thought to reflect walking during daily activities of living more appropriately. We will be using that test. We will be using treadmill testing, and we will also be using various measurements of functional capacity.

Dr George Andros (Encino, Calif): I had a few questions. Isn’t this basically a pharmacology investigation?

Dr Killewich: Okay.

Dr Andros: And in most pharmacology investigations there are at least two essential criteria for the assessment of the drug; one is a time curve for the appearance of the effect, and a second is a dose-response curve. Have you inquired into these?

Several years ago, we were involved with the use of a magnetic resonance flowmetry technique and we had a lengthy discussion with Gene Strandness about whether or not blood flow at rest in claudicants is increased or decreased or the same, and our data showed that blood flow as compared to calf plethysmography was decreased in claudicants at rest. He felt that it was not decreased, and we had many discussions on this. So, there is a fundamental question here: is blood flow in claudicants decreased or normal?

Then there is the other question of biopsy distortion. In my previous life as a biophysicist, I did many animal studies on biopsy distortion of blood flow and we found that is a significant effect on blood flow as you do serial biopsies of muscle structure.

The final point that I had was that I think there is a missing control group. You have a control group that is given phenylalanine and a normal group. Do you have a control group in which you have claudicants with their presumed reduced blood flow who received phenylalanine and those who received some other nonessential amino acid or some other control group within the group that you are studying so that the control group I believe should be two different claudicant groups rather than a claudicant and a control group.

Dr Killewich: Thank you very much, Dr Andros. Yes, we have done the time study and also the dose-response study, and we have done those at a couple different doses of amino acids. We did it at 8 grams of essential amino acids. We did it at 15 grams. We have also done nonessential amino acids and amino acid and carbohydrate combinations. Yes, we did do those and we found that the most benefit was associated with the 15-gram essential amino acid in this particular time duration.

As far as is blood flow reduced at rest, I do not really know the answer to that question other than what we did in this study and that is we measured calf blood flow using strain gauge and that is a pretty standard technique which is used in the literature. A lot of studies have used that technique and so all I can say is that we did find it was reduced at rest in these patients.

As far as biopsy distortion of blood flow, I think the key point there is that we measured blood flow at the end of the protocol after we had already done three muscle biopsies and so you could make the argument that—I think it is what you are saying—the biopsies distorted the blood flow measurement which we did after the biopsies, but in fact, when we started doing this we were doing blood flow measurements at the beginning of the protocol and we did them twice, once at the beginning and once at the end. We did not find any difference and so we stopped doing them at the beginning because it was too cumbersome for the overall protocol.

Finally, I would have to say that you are correct, we do not have the control group where we gave them a different amino acid.

Dr Niren Angle (San Diego, Calif): Beautiful paper. I have a quick question for you. If you look in the exercise physiology literature there is a fair amount of data that says that most protein does not matter. It is the branch chain amino acid supplementation, the isolucine, the valine, and the lucine, and I know there is a fair amount of controversy about that, but could you comment on using just branch chain amino acids instead of essential.

Number two, have you entertained doing this with having them do resistance training or exercising and then supplementing them within a few hours or within an hour of exercise because that seems to increase muscle mass much more than the standard supplementation of protein at any variable time during the day.

Thank you.

Dr Killewich: Thank you very much. I cannot say that we have actually studied branch chain amino acids and so I cannot say whether they would have the same benefit that our particular formulation of essential amino acids has but you certainly have a valid point there.

As far as using exercise training, in particular endurance training, as well as amino acids; yes, we have contemplated doing that. We have done some studies on that, not in claudicants, and we would like to do that in the future. Other investigators in Dr Wolfe’s group have shown that the combination of endurance exercise and oral amino acids will actually improve your muscle protein synthesis and your strength greater than just amino acids alone. Needless to say, the question is always raised with claudicants as to how much they can exercise, but I chose amino acids initially because I felt that this might be more widely applicable.