Sodium nitrate co-ingestion with protein does not augment postprandial muscle protein synthesis rates in older, type 2 diabetes patients

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Kouw IW, Cermak NM, Burd NA, Churchward-Venne TA, Senden JM, Gijsen AP, van Loon LJ. Sodium nitrate co-ingestion with protein does not augment postprandial muscle protein synthesis rates in older, type 2 diabetes patients. Am J Physiol Endocrinol Metab 311: E325–E334, 2016. First published May 24, 2016; doi:10.1152/ajpendo.00122.2016.—The age-related anabolic resistance to protein ingestion is suggested to be associated with impairments in insulin-mediated capillary recruitment and postprandial muscle tissue perfusion. The present study investigated whether dietary nitrate co-ingestion with protein improves muscle protein synthesis in older, type 2 diabetes patients. Twenty-four men with type 2 diabetes (72 ± 1 yr, 26.7 ± 1.4 m/kg² body mass index, 7.3 ± 0.4% HbA1c) received a primed continuous infusion of L-[ring-2H₅]phenylalanine and L-[1-¹³C]leucine and ingested 20 g of intrinsically L-[1-¹³C]phenylalanine- and L-[1-¹³C]leucine-labeled protein with (PRONO3) or without (PRO) sodium nitrate (0.15 mmol/kg). Blood and muscle samples were collected to assess protein digestion and absorption kinetics and postprandial muscle protein synthesis rates. Upon protein ingestion, exogenous phenylalanine appearance rates increased in both groups (P < 0.001), resulting in 55 ± 2% and 53 ± 2% of dietary protein-derived amino acids becoming available in the circulation over the 5h postprandial period in the PRO and PRONO3 groups, respectively. Postprandial myofibrillar protein synthesis rates based on L-[ring-2H₅]phenylalanine did not differ between groups (0.025 ± 0.004 and 0.021 ± 0.007%/h over 0–2 h and 0.032 ± 0.004 and 0.030 ± 0.003%/h over 2–5 h in PRO and PRONO3, respectively, P = 0.7). No differences in incorporation of dietary protein-derived L-[1-¹³C]phenylalanine into de novo myofibrillar protein were observed at 5 h (0.016 ± 0.002 and 0.014 ± 0.002 mole percent excess in PRO and PRONO3, respectively, P = 0.8). Dietary nitrate co-ingestion with protein does not modulate protein digestion and absorption kinetics, nor does it further increase postprandial muscle protein synthesis rates or the incorporation of dietary protein-derived amino acids into de novo myofibrillar protein in older, type 2 diabetes patients.

dietary nitrate; protein ingestion; type 2 diabetes; anabolic resistance

AGING IS ASSOCIATED with loss of skeletal muscle mass and strength, termed sarcopenia, and results in loss of functional capacity, a higher risk for hospitalization, and development of chronic metabolic diseases (33). Recently, we (20, 31), as well as others (25, 35), showed that sarcopenia is more pronounced in older individuals with type 2 diabetes than in age-matched, normoglycemic controls. Since skeletal muscle tissue is a large site of postprandial glucose disposal, it is evident that the age-related loss of skeletal muscle mass is often accompanied by an insulin-resistant state (20, 31, 35).

Maintenance of skeletal muscle mass is regulated by muscle protein synthesis and muscle protein breakdown rates. A chronic imbalance between muscle protein synthesis and muscle protein breakdown rates contributes to the accelerated loss of skeletal muscle mass with aging. It has been well established that protein ingestion and the associated increase in postprandial plasma amino acids in the circulation directly increase muscle protein synthesis rates (15, 26, 34, 50). However, the postprandial stimulation of muscle protein synthesis following protein intake is impaired with aging, a phenomenon commonly referred to as anabolic resistance (15). This anabolic resistance to protein intake is considered to be a key factor in the progressive loss of skeletal muscle mass with aging. Although the factor(s) underpinning anabolic resistance remains unclear, age-related impairments in endothelium-dependent vasodilation and blood flow in response to nutrient (32) and exercise (19) stimuli are suggested to play a key role.

The postprandial muscle protein synthetic response to protein feeding is regulated by numerous factors, such as protein digestion and amino acid absorption, amino acid availability in the circulation, delivery to skeletal muscle tissue, and uptake in the muscle (9). The postprandial increase in muscle microvascular perfusion facilitates the delivery of amino acids, nutrients, and growth factors to the muscle, thereby allowing muscle protein synthesis rates to increase after protein ingestion (43, 49). However, aging and insulin resistance are associated with reduced skeletal muscle capillary density, impaired endothelial function, and reduced nitric oxide (NO) availability (14, 20, 32, 39, 40). As such, age-related impairments in vascular function due to an impaired NO-mediated vasodilatory response and decreased muscle tissue perfusion may diminish feeding-mediated increases in muscle protein synthesis and, thus, contribute to the anabolic resistance of aging (7, 24).

It has been reported that dietary nitrate (NO₃⁻) supplementation, via sequential reduction of NO₃⁻ to nitrite (NO₂⁻) and NO, can stimulate NO synthesis and increase NO bioavailability (55). Moreover, it has been suggested that NO has a role in promoting insulin delivery to skeletal muscle tissue and increasing insulin-stimulated muscle perfusion and capillary action (22, 51). As such, ingestion of a single dose of dietary NO₃⁻ might increase endothelial NO availability, thereby increasing blood flow and tissue perfusion and the subsequent muscle anabolic response to food intake. Recently, the acute ingestion of NO₃⁻-rich dietary sources has been shown to improve vascular function (21, 23, 29, 53) and macro- and microvascular blood flow (3, 29) in healthy subjects, as well as...
in overweight individuals and diabetes patients. In support of these findings, pharmacological increases in blood flow and tissue perfusion following administration of a NO-donating vasodilator, sodium nitroprusside, combined with amino acid provision have been shown to increase postprandial muscle protein synthesis rates in older subjects (17). However, whether dietary NO\textsubscript{3} co-ingestion with a protein meal can be used as a nutritional strategy to augment the postprandial muscle protein synthetic response remains to be assessed.

In particular, dietary NO\textsubscript{3} may serve as an effective nutritional strategy to counteract the age-related anabolic resistance of muscle protein synthesis to protein ingestion through NO-mediated increases in skeletal muscle microvascular perfusion. In the current investigation we hypothesized that a single dose of dietary NO\textsubscript{3} co-ingested with protein increases amino acid delivery to skeletal muscle tissue and, subsequently, enhances the postprandial muscle protein synthetic response.

METHODS

Subjects. Twenty-four older (72 ± 1 yr), type 2 diabetes patients (body mass index = 26.7 ± 1.4 kg/m\textsuperscript{2}, Hb\textsubscript{A\textsubscript{1C}} = 7.3 ± 0.4%) were randomly assigned to two groups: a group that ingested protein with NaCl (PRO) or a group that ingested protein with NaNO\textsubscript{3} (PRONO3). Subjects’ characteristics are presented in Table 1. All subjects were using oral blood glucose-lowering medication and continued their habitual medication. Diabetes patients had been diagnosed with type 2 diabetes 2–18 yr prior to screening. One subject in the PRO group was unable to complete the trial and was, therefore, excluded from analysis. Subjects were informed of the nature and possible risks of the experimental procedures prior to providing written informed consent. The trial, conducted between August 2011 and July 2012 at Maastricht University, was part of a larger project investigating the impact of protein feeding on muscle metabolism in older, type 2 diabetes patients (28). The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre and conformed to standards for the use of human subjects in research as outlined in the latest version of the Declaration of Helsinki.

Table 1. Subjects’ characteristics

<table>
<thead>
<tr>
<th>Intervention</th>
<th>PRO (n = 11)</th>
<th>PRONO3 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>71 ± 1</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>80.4 ± 1.6</td>
<td>83.0 ± 1.8</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
<td>26.2 ± 0.5</td>
<td>27.3 ± 0.6</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>60.6 ± 1.0</td>
<td>60.8 ± 1.4</td>
</tr>
<tr>
<td>Appendicular lean mass, kg</td>
<td>25.8 ± 0.6 (32%)</td>
<td>25.1 ± 0.8 (30%)</td>
</tr>
<tr>
<td>Skeletal mass index, kg/m\textsuperscript{2}</td>
<td>8.4 ± 0.2</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Fat, %</td>
<td>21.8 ± 0.8</td>
<td>23.6 ± 0.8</td>
</tr>
<tr>
<td>Basal plasma glucose, mmol/l</td>
<td>9.6 ± 0.9</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>Plasma glucose (OGTT at t = 120 min), mmol/l</td>
<td>16.3 ± 0.9</td>
<td>17.9 ± 0.9</td>
</tr>
<tr>
<td>Basal plasma insulin, mU/l</td>
<td>16.8 ± 2.4</td>
<td>17.4 ± 1.6</td>
</tr>
<tr>
<td>Plasma insulin (OGTT at t = 120 min), mU/l</td>
<td>45.6 ± 8.0</td>
<td>42.3 ± 7.1</td>
</tr>
<tr>
<td>Hb\textsubscript{A\textsubscript{1C}}, %</td>
<td>7.1 ± 0.3</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>OGIS, ml-min\textsuperscript{-1}·m\textsuperscript{-2}</td>
<td>286 ± 11</td>
<td>260 ± 9</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.5 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. PRO, protein with NaCl coingestion; PRONO3, protein with NaNO\textsubscript{3} coingestion; BMI, body mass index; Hb\textsubscript{A\textsubscript{1C}}, glycosylated hemoglobin; OGTT, oral glucose tolerance test; OGIS, oral glucose insulin sensitivity; HOMA-IR: homeostasis model assessment of insulin resistance. Data were analyzed by unpaired Student’s t-test.

Pretesting. All subjects participated in a prescreening session to assess their Hb\textsubscript{A\textsubscript{1C}} glucose tolerance [by a 2-h oral glucose tolerance test (2)], blood pressure, weight, height, and body composition [by dual-energy X-ray absorptiometry (Discovery A, Hologic, Bedford, MA)]. The subjects were deemed healthy on the basis of their responses to a medical questionnaire and screening results. All subjects were instructed to refrain from strenuous physical activity and to maintain their diet as constant as possible for 2 days prior to the experimental infusions. On the evening before the trial, the subjects consumed a standardized meal (2.38 MJ, 31.7 ± 1.1 kJ/kg body wt) composed of 15 energy percent (en%) protein, 55 en% carbohydrate, and 30 en% fat.

Experimental protocol. At 0800, after an overnight fast, subjects arrived at the laboratory by car or public transport. Upon the subjects’ arrival at the laboratory, a Teflon catheter was inserted into an antecubital vein for infusion and a second cannula was inserted into the dorsal hand vein of the contralateral arm, which was placed in a hot box (60°C) for arterialized blood sampling (1). After baseline blood collection (−210 min), the plasma phenylalanine, tyrosine, and leucine pools were primed with a single intravenous (priming) dose of l-[ring-\textsuperscript{2H\textsubscript{5}}]phenylalanine (2 mmol/kg), l-[ring-\textsuperscript{2H\textsubscript{5}}]tyrosine (0.615 mmol/kg), and l-[1-\textsuperscript{13C}]leucine (4.0 mmol/kg). Subsequently, the continuous infusion (0.05 mmol·kg\textsuperscript{-1}·min\textsuperscript{-1} for l-[ring-\textsuperscript{2H\textsubscript{5}}]phenylalanine, 0.015 mmol·kg\textsuperscript{-1}·min\textsuperscript{-1} for l-[ring-\textsuperscript{2H\textsubscript{5}}]tyrosine, and 0.100 mmol·kg\textsuperscript{-1}·min\textsuperscript{-1} for l-[1-\textsuperscript{13C}]leucine) was initiated and maintained for 8.5 h. At −120 min, subjects ingested a single bolus of NaNO\textsubscript{3} [0.15 mmol/kg body wt, which provided NO\textsubscript{3} at 12.75 mg/kg body wt (PRONO3 group; BASF, Ludwigshafen, Germany)] or an equimolar amount of NaCl dissolved in 250 ml of water (PRO group; Glacía British salt, United Kingdom) dissolved in 250 ml of water. We provided a single dose of dietary NO\textsubscript{3} at 2 h prior to protein ingestion to ensure that the increase in NO\textsubscript{3}/NO\textsubscript{2} concentrations would correspond with the postprandial increase in plasma amino acid concentrations (58). Blood samples were collected during the infusion at −120, −90, −60, −30, 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min relative to the intake of the protein drink. Blood samples were collected into EDTA-containing tubes and centrifuged at 1,000 g at 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. Muscle biopsies were collected for determination of postabsorptive muscle protein synthesis rates (−120 and 0 min). Immediately after the second biopsy, subjects ingested a single bolus of 20 g of intrinsically l-[1-\textsuperscript{13C}]phenylalanine- and l-[1-\textsuperscript{13C}]leucine-labeled casein protein dissolved in water that was flavored with 1.5 ml of vanilla (IMCD Benelux, Rotterdam, The Netherlands). Additional biopsies (from the contralateral leg) were collected at 120 and 300 min for measurement of postprandial muscle protein synthesis rates. The Bergström needle technique (5) was used to collect muscle biopsies from the middle region of the vastus lateralis (15 cm above the patella). All biopsy samples were freed from visible adipose tissue and blood, immediately frozen in liquid nitrogen, and stored at −80°C until subsequent analysis.

Production of intrinsically labeled protein. Intrinsically l-[1-\textsuperscript{13C}]phenylalanine- and l-[1-\textsuperscript{13C}]leucine-labeled casein was obtained by infusion of a lactating Holstein cow with l-[1-\textsuperscript{13C}]phenylalanine (455 mmol/min) and l-[1-\textsuperscript{13C}]leucine (200 mmol/min), collection of the milk, and purification of the casein fraction, as described previously (8, 36, 45). Enrichments of l-[1-\textsuperscript{13C}]phenylalanine and l-[1-\textsuperscript{13C}]leucine in the casein were 38.7 and 9.3 mole percent excess (MPE), respectively. Participants received a beverage containing 20 g of casein (containing 1.54 g of leucine) in a total volume of 350 ml. The casein protein met all chemical and bacteriological specifications for human consumption.

Plasma analyses. Plasma NO\textsubscript{3} and NO\textsubscript{2} were analyzed by gas-phase chemiluminescence analysis, which has been described previously (12). Briefly, plasma NO\textsubscript{3} and NO\textsubscript{2} concentrations are determined by their reduction to NO. The spectral emission of electronically excited nitrogen dioxide, from the NO reaction with ozone, is detected by a thermoelectrically cooled, red-sensitive photomultiplier.
tube, housed in a gas-phase chemiluminescence NO analyzer (Sievers NOA 280; Analytix). Plasma glucose and insulin concentrations were analyzed using commercially available kits [GLUC3 (catalog no. 05168791 190, Roche) and ImmunoLogic (catalog no. 12017547 122, Roche), respectively]. Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry (GC-MS) analysis (model 7890A/GC/5975C/MSD, Agilent, Little Falls, VA). Specifically, internal standards of [U-13C6]leucine, [U-13C815N]phenylalanine, and [U-13C615N]tyrosine were added to the samples, which were deproteinized on ice with dry 5-sulfosalicylic acid. Free amino acids were purified using cation-exchange chromatography (AG 50W-X8 resin, 100- to 200-μm dry mesh; Bio-Rad Laboratories, Hercules, CA) and converted to their tert-butyl dimethylsilyl (t-BDMS) derivative before GC-MS analysis. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass-to-charge ratios (m/z) of 302 and 308 for unlabeled and [U-13C6]labeled leucine, respectively, m/z 336 and 346 for unlabeled and [U-13C815N]-labeled phenylalanine, respectively, and m/z 466 and 476 for unlabeled and [U-13C615N]-labeled tyrosine, respectively. The plasma phenylalanine and tyrosine 13C and 2H enrichments were determined using selective ion monitoring at m/z 336, 337, and 341 for unlabeled and labeled (1-13C and ring-2H2) phenylalanine, respectively, and m/z 466, 467, 468, and 470 for unlabeled and labeled (1-13C, ring-3,5-2H2, and ring-2H4) tyrosine, respectively. For 1-[U-13C6]leucine, m/z 302 (m + 0) and m/z 303 (m + 1) were monitored. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation that may have occurred during the analysis. Phenylalanine and tyrosine enrichments were corrected to measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation that may have occurred during the analysis.

**Calculations.** Whole body amino acid kinetics in non-steady-state conditions were calculated from ingestion of L-[1-13C]phenylalanine-labeled protein, intravenous infusion of L-[ring-2H5]phenylalanine and L-[ring-3,5-2H2]tyrosine, and arterialized venous blood sampling. Total, endogenous, and endogenous phenylalanine appearance rates (Rao, in μmol Phe kg⁻¹ min⁻¹) and plasma phenylalanine availability [i.e., the fraction of dietary protein-derived phenylalanine that appeared in the systemic circulation (Pheplasma)] were calculated using modified Steele’s equations (6, 16, 57).

**Muscle analyses.** The muscle intracellular free amino acids were extracted from ~50 mg of wet muscle tissue. The muscle was homogenized on ice with a Teflon-coated pestle using ice-cold 2% perchloric acid (7 μl wet weight) and then centrifuged at 10,000 g for 4°C for 5 min. The supernatant was collected, and this process was repeated twice. The supernatant was used as the muscle intracellular free amino acid pool and processed in the same manner as the plasma samples for isotopic enrichment by GC-MS analysis. Myofibrillar protein-enriched fractions were extracted from a separate ~50mg piece of wet muscle tissue by hand-homogenization on ice using a pestle (Teflon) in a standard extraction buffer (7.5 μl/mg) (27). The samples were spun at 1,500 g and 4°C for 10 min. The supernatants containing sarcoplasmic proteins were removed. The remaining myofibrillar and collagen pellets were washed with 500 μl of extraction buffer and spun at 700 g and 4°C for 10 min. The myofibrillar protein was solubilized by addition of 1.5 ml of 0.3 M NaOH and heating at 50°C for 30 min, with vortex mixing every 10 min. Samples were centrifuged at 10,000 g and 4°C for 5 min, and the supernatant containing the myofibrillar-enriched fraction was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by addition of 1 ml of 1 M perchloric acid and spinning at 700 g and 4°C for 10 min. The myofibrillar protein was washed twice with 70% ethanol. Amino acids were liberated by addition of 2 ml of 70% ethyl alcohol and heating at 110°C for 20 h. The hydrolyzed myofibrillar protein fraction was dried under a nitrogen stream while being heated to 120°C and, subsequently, dissolved in a 25% acetic acid solution and passed over a Dowex exchange resin (AG 50W-X8, 100- to 200-μm mesh hydrogel form; Bio-Rad Laboratories). The free amino acids were eluted with 2 M NH4OH and dried, and the purified amino acids were derivatized into N-tert-butylmethylsilyl-N-methyltrifluoroaceticamide-phenylglycine (11, 38) for measurement of L-[ring-2H5]phenylalanine labeling using GC-MS analysis, as described previously (10). To reduce the signal-to-noise ratio during GC-MS analysis at low tracer enrichments, the phenylalanine from the myofibrillar protein hydrolysates was enzymatically decarboxylated to phenylethylamine (9) prior to r-BDMS derivatization (32, 33). Enrichments of the protein-bound samples were determined by selected ion monitoring for phenylalanine m/z 183 (m + 5) to m/z 180 (m + 2) and a single linear standard curve (to avoid slope influences on the measured tracer-to-tracee ratio) from mixtures of known m + 5 to m + 0 ratios. To avoid saturation of the mass spectrometer and eliminate bias due to any potential concentration dependencies (22), the split ratio was adjusted prior to the injection of each sample, so that nearly equal amounts of phenylalanine were injected for all samples and standards. The derivatized amino acids were separated on a 30 m × 0.25 mm × 0.50 μm HP-5MS column (temperature program: 80°C for 1 min, 30°C/min ramp to 300°C, hold for 10 min). Separate aliquots of the purified amino acids were also converted to their N(3,5)-ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate for determination of the L-[1-13C6]phenylalanine and L-[1-13C6]leucine labeling of the myofibrillar proteins by gas chromatography combustion-isotope ratio mass spectrometry analysis (GC-I-RMS Trace GC Ultra, model MAT 253, Thermo Scientific, Bremen, Germany). Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation that may have occurred during the analysis.

The plasma phenylalanine enrichment of the oral tracer, dEpo/dt, was calculated using modified Steele’s equations (6, 16, 57).

\[
\text{Total phenylalanine } R_o = \frac{F_{iv} - \left[ pV \cdot C(t) \cdot \frac{dE_{po}}{dt} \right]}{E_{po}(t)} \quad (1)
\]

Total phenylalanine Ra was calculated using Eq. 1, where Fiv is the intravenous tracer infusion rate (in μmol kg⁻¹ min⁻¹) and pV is the distribution volume for phenylalanine (0.125 l/kg) (6). C(t) is the mean plasma phenylalanine concentration between two consecutive time points, and dEpo/dt represents the time-dependent change in plasma phenylalanine enrichment derived from the intravenous tracer and Epo(t) is the mean plasma phenylalanine enrichment from the intravenous tracer between two consecutive time points.

\[
\text{exogenous } R_e = \frac{\int_{t_1}^{t_2} pV \cdot C(t) \cdot \frac{dE_{po}}{dt} \, dt}{E_{po}(t)} \quad (2)
\]

Exogenous phenylalanine Re, (Eq. 2; Exo Ra) represents the plasma entry rate of dietary protein-derived phenylalanine. Epo(t) is the mean plasma phenylalanine enrichment of the oral tracer, dEpo/dt represents the time-dependent change in plasma phenylalanine enrichment derived from the oral tracer, and Epo(t) is the enrichment of L-[1-13C6]phenylalanine in the dietary protein.

\[
\text{endogenous } R_a = \text{total } R_a - \text{exogenous } R_e = F_{iv} \quad (3)
\]

Endogenous phenylalanine Ra (Eq. 3; Endo Ra) represents the plasma entry rate of phenylalanine derived from whole body protein breakdown.

\[
\text{pheplasma} = \frac{\text{AUC}_{\text{Exo}R_e}}{\text{AUC}_{\text{Endo}R_a}} \cdot \text{BW} \cdot 100\% \quad (4)
\]
Plasma analyses. Plasma NO$_3^-$ and NO$_2^-$ concentrations are presented in Fig. 1. Plasma NO$_3^-$ concentrations (Fig. 1A) did not differ at baseline (~210 min) between intervention groups and increased following NaNO$_3^-$ ingestion (P < 0.001). Throughout the experiment, NO$_3^-$ concentrations were significantly higher in PRONO3 than the PR0 group (P < 0.001, time × intervention interaction P < 0.001, iAUC P < 0.001), reaching peak values of 663 ± 22 and 39 ± 2 μmol/l in the PRONO3 and PRO groups, respectively (P < 0.001). Plasma NO$_2^-$ concentrations (Fig. 1B) increased significantly following NaNO$_3^-$ ingestion in the PRONO3 group only (time effect P < 0.01, group effect P < 0.05, time × intervention interaction P < 0.01), reaching peak values of 788 ± 138 and 389 ± 55 mmol/l in the PRONO3 and PRO groups, respectively (peak values P < 0.05, iAUC P < 0.01).

Plasma glucose and insulin concentrations are shown in Fig. 2. Plasma glucose concentrations (Fig. 2A) did not differ between interventions throughout the experimental trial. Plasma insulin concentrations (Fig. 2B) increased following protein ingestion (P < 0.001), but to a greater extent in the PRO than the PRONO3 group (time × intervention interaction P < 0.001, iAUC P = 0.4).

Plasma amino acid concentrations (Fig. 3) increased after protein ingestion (P < 0.001), with no differences between groups for phenylalanine (Fig. 3A) and leucine (Fig. 3C) concentrations. Plasma tyrosine concentrations (Fig. 3B) were higher in the PRO group during the postprandial period (intervention effect P < 0.05, iAUC P = 0.3).

Plasma amino acid enrichments are presented in Fig. 4. Plasma L-[ring-$^{2}$H$_{5}$]phenylalanine (Fig. 4A) and L-[1-$^{13}$C]leucine (Fig. 4C) enrichments did not differ between interventions in the basal and postprandial periods. Plasma L-[1-$^{13}$C]phenylalanine enrichments (Fig. 4B) increased following protein
ingestion in both intervention groups (P < 0.001), reaching peak values of 12.3 ± 0.7 MPE at 72 ± 12 min and 11.8 ± 0.8 MPE at 86 ± 24 min in the PRO and PRONO3 groups, respectively, and did not differ between groups (intervention effect P = 0.7, iAUC P = 0.8).

Whole body phenylalanine kinetics. Whole body phenylalanine Ra values are presented in Fig. 5. Exogenous phenylalanine Ra (Fig. 5A) increased rapidly following protein ingestion (P < 0.001) and did not differ between groups. The fraction of dietary protein-derived phenylalanine that was released in the plasma over the 5h postprandial period was 55 ± 2% and 53 ± 2% in the PRO and PRONO3 groups, respectively. Endogenous phenylalanine Ra (Fig. 5B) decreased following protein ingestion and did not differ between intervention groups. No differences were detected between interventions in total plasma phenylalanine Ra (Fig. 5C) and Ru (Fig. 5D).

Postabsorptive and postprandial whole body protein breakdown, synthesis, oxidation, and net balance are presented in Fig. 6. Whole body protein breakdown rates decreased following protein ingestion (P < 0.001). Whole body protein synthesis rates increased after protein ingestion (P < 0.001), with no differences between groups (P = 0.3). Whole body protein oxidation rates were significantly higher in the PRO than the PRONO3 group in the basal and postprandial periods (P < 0.05). Whole body protein net balance in the postabsorptive phase was significantly higher in the PRONO3 than the PRO group (0.66 ± 0.10 vs. 1.04 ± 0.12 µmol·kg·h⁻¹, P < 0.05) and increased in the postprandial phase (7.71 ± 0.33 and 7.80 ± 0.53 µmol·kg·h⁻¹ in PRO and PRONO3, respectively, P < 0.001), with no differences between groups (P = 0.8).

Muscle protein synthesis. Myofibrillar protein synthesis rates, expressed as FSR, are presented in Fig. 7. Postabsorptive myofibrillar FSR based on the l-[ring-2H₅]phenylalanine tracer (Fig. 7A), with plasma l-[ring-2H₅]phenylalanine enrichments used as precursor pool, did not differ between interventions (P = 0.6). After protein ingestion, myofibrillar protein synthesis rates in the PRO and PRONO3 groups averaged 0.025 ±

![Fig. 2. Plasma glucose (A) and insulin (B) concentrations in older, type 2 diabetes patients following protein ingestion with (PRONO3, n = 12) or without (PRO, n = 11) dietary NO₃. Vertical lines indicate ingestion of the drinks: dotted lines, NaNO₃ or NaCl; dashed lines, 20 g protein. Values are means ± SE. Data were analyzed with repeated-measures (time × intervention) ANOVA.](https://doi.org/10.1152/ajpendo.00122.2016)

![Fig. 3. Plasma phenylalanine (A), tyrosine (B), and leucine (C) concentrations in older, type 2 diabetes patients following protein ingestion with (PRONO3, n = 12) or without (PRO, n = 11) dietary NO₃. Vertical lines indicate ingestion of the drinks: dotted lines, NaNO₃ or NaCl; dashed lines, 20 g protein. Inset: iAUC. Values are means ± SE. Data were analyzed with repeated-measures (time × intervention) ANOVA.](https://doi.org/10.1152/ajpendo.00122.2016)
PRONO3 groups averaged 0.039 ± 0.007 and 0.040 ± 0.011%/h and 0.046 ± 0.008 and 0.038 ± 0.007%/h, respectively, with no differences between groups (time effect \( P = 0.7\), intervention effect \( P = 0.7\)). Myofibrillar protein-bound \( L-[1^{-13}C]\)phenylalanine enrichments in PRO and PRONO3 groups averaged 0.0051 ± 0.0011 and 0.0061 ± 0.0013 MPE, respectively, at 2 h and 0.0155 ± 0.0020 and 0.0135 ± 0.0022 MPE, respectively, at 5 h, with no differences between groups (time effect \( P < 0.001\), intervention effect \( P = 0.8\)).

Muscle tissue free \( L-[ring-2H_5]\)phenylalanine, \( L-[1^{-13}C]\)leucine, and \( L-[1^{-13}C]\)phenylalanine enrichments did not differ between groups (Table 2). Similar observations were observed when myofibrillar protein synthesis rates were calculated based on use of muscle free phenylalanine enrichments as precursor pool (data not shown).

**DISCUSSION**

This is the first study to assess if a single dose of dietary \( NO_3^-\) ingested with protein increases amino acid delivery to skeletal muscle tissue and, subsequently, augments the postprandial muscle protein synthetic response in older, type 2 diabetes patients. The ingestion of 20 g of protein increased plasma amino acid availability (Figs. 3–5), stimulated whole body protein synthesis (Fig. 6), increased myofibrillar protein synthesis rates (Fig. 7), and enhanced the use of dietary protein-derived amino acids for de novo muscle protein synthesis (Fig. 8). However, co-ingestion of a single dose of dietary \( NO_3^-\) with the same amount of protein did not modulate postprandial protein handling in older men with type 2 diabetes.

Reductions in vascular function in response to aging and/or diabetes may be due in part to reductions in \( NO_3^-\) synthesis and a reduced \( NO\)-mediated vasodilatory response to feeding-induced hyperinsulinemia (24). Consequently, a reduction in \( NO\)-mediated vascular function may attenuate postprandial plasma amino acid delivery to tissues, thereby having a negative impact on normal feeding-mediated increases in postprandial muscle protein synthesis rates. Current evidence suggests that the ingestion of dietary \( NO_3^-\) can substantially increase \( NO\) availability, which may be of particular importance in pathological conditions associated with a disrupted \( NO\) synthase (NOS)-NO pathway, as in the case of aging or diabetes (22, 54). Inorganic \( NO_3^-\) is converted to \( NO_2^-\) by \( NO_2^-\) reductase-expressing bacteria, which naturally reside in the oral cavity. Once salivary \( NO_3^-\) is swallowed, some of it enters the systemic circulation, where it is chemically reduced to \( NO\). A recent study by Wylie and co-workers (58) showed that changes in plasma \( NO_2^-\) occur 2–3 h after ingestion of beetroot juice (a concentrated source of dietary \( NO_3^-\)). Therefore, we provided a single dose of dietary \( NO_3^-\) at 2 h prior to protein ingestion, so that increased \( NO_2^-\) concentrations would temporally correspond with increased postprandial plasma amino acid concentrations. The ingestion of \( NaNO_3\) increased plasma \( NO_3^-\) concentrations, which remained elevated for up to 7 h (Fig. 1). Moreover, plasma \( NO_2^-\) increased following the ingestion of \( NaNO_3\), reaching peak concentrations of ~800 mmol/l at 30 min after protein ingestion. These findings indicate an intact enterosalivary circuit in older, type 2 diabetes patients.
After protein ingestion, plasma amino acid concentrations increased in both groups (Fig. 3). By combining the ingestion of intrinsically 1-[1-13C]phenylalanine-labeled protein with a primed continuous 1-[ring-2H5]phenylalanine infusion, we were able to measure protein-derived 1-[1-13C]phenylalanine enrichments (Fig. 4) and quantify the Ra of dietary protein-derived amino acids into the circulation (Fig. 5) (8, 45). Plasma 1-[1-13C]phenylalanine enrichments increased directly following protein ingestion in both groups, reaching peak values of 12.3 ± 0.7 and 11.8 ± 0.8 MPE in the PRO and PRONO3 groups, respectively. In total, 55 ± 2% and 53 ± 2% of the ingested protein became available in the circulation during the 5-h postprandial period in the PRO and PRONO3 groups, respectively. Despite previous reports that dietary strategies augmenting NO metabolism may alter gut and organ tissue perfusion (47, 56), the greater plasma NO2 availability in the PRONO3 group did not modulate dietary protein digestion, amino acid absorption, or the amount of protein-derived amino acids that became available within the circulation after the ingestion of 20 g of protein in these older, type 2 diabetes patients.

Protein ingestion increased postprandial whole body protein synthesis and decreased whole body protein breakdown rates, resulting in a positive postprandial whole body protein net balance. However, ingestion of dietary NO3 did not modulate postprandial whole body protein turnover rates (Fig. 6). Our results seem to agree with previous observations by Thibault and colleagues (42), who were unable to detect an effect of citrulline, a substrate for NOS-mediated NO synthesis, on whole body protein turnover. In addition to measuring whole body protein turnover rates, we also measured the impact of dietary NO3 ingestion on the postprandial myofibrillar protein synthetic response. The ingestion of a meal-like amount of protein increased postprandial myofibrillar protein synthesis rates; however, co-ingestion of dietary NO3 did not further increase postprandial myofibrillar protein synthesis rates (Fig. 7). In addition to determining the postprandial myofibrillar protein synthesis rates, we used highly enriched (38.7 MPE), intrinsically 1-[1-13C]phenylalanine-labeled protein to assess the metabolic fate of the ingested protein by measuring the incorporation of dietary NO3 into amino acids that became available to the circulation after the ingestion of 20 g of protein.
DIETARY NITRATE CO-INGESTION AND MUSCLE PROTEIN SYNTHESIS

Fig. 7. Myofibrillar muscle protein fractional synthesis rates (FSR) in older, type 2 diabetes patients intravenously infused with L-[ring-2H5]phenylalanine (A) and L-[1-13C]leucine (B) following 20 g protein ingestion with (PRONO3, n = 12) or without (PRO, n = 11) dietary NO3. Values are means ± SE. Data were analyzed with unpaired Student’s t-test (between groups) and repeated-measures (time × intervention) ANOVA. FSR based on L-[ring-2H5]phenylalanine enrichments: time effect P = 0.03, intervention effect P = 0.7; FSR based on L-[1-13C]leucine enrichments: time effect P = 0.7, intervention effect P = 0.05. *Significant time effect, P < 0.05.

Fig. 8. Myofibrillar protein-bound L-[1-13C]phenylalanine enrichments in older, type 2 diabetes patients following 20 g protein ingestion with (PRONO3, n = 12) or without (PRO, n = 11) dietary NO3. Values are means ± SE. Data were analyzed with unpaired Student’s t-test (between groups) and repeated-measures (time × intervention) ANOVA. Time effect P < 0.001, intervention effect P = 0.8. *Significant time effect, P < 0.001.

Reductions in NO synthesis in older adults may be partly responsible for diminished NO-mediated capillary recruitment and the blunted muscle protein synthetic response to the postprandial rise in plasma amino acid concentrations. In support of this notion, pharmacological strategies utilizing NO donors (i.e., sodium nitroprusside or methacholine) have been shown to effectively increase blood flow and microvascular perfusion and augment the muscle protein synthetic response to amino acid administration (17, 44). However, practical dietary interventions providing precursors for endothelial NOS-mediated NO synthesis, such as citrulline (13) and/or arginine (41) co-ingested with protein, have not been effective in increasing the postprandial rise in muscle protein synthesis rates. In the present study, dietary NO3 was provided as a practical nutritional strategy to increase NO availability through a separate pathway that does not require endothelial NOS. Supplementation of dietary NO3 has been shown to have significant effects on endothelial function (21), blood pressure (4, 53, 58), and O2 consumption during exercise (58) in young and older subjects. However, despite the established effects of NO3 on vascular function, our results demonstrate that co-ingestion of NO3 with a meal-like amount of protein does not augment postprandial plasma amino acid availability, modulate whole body protein metabolism, increase skeletal muscle protein synthesis

Table 2. Muscle tissue free enrichments

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>0 h</th>
<th>2 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[ring-2H5]phenylalanine, MPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PRO</td>
<td>11</td>
<td>4.37 ± 0.42</td>
<td>5.50 ± 0.38</td>
<td>5.00 ± 0.26</td>
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<td>PRONO3</td>
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<td>5.31 ± 0.48</td>
<td>5.14 ± 0.28</td>
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<tr>
<td>L-[1-13C]leucine, MPE</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRO</td>
<td>11</td>
<td>3.51 ± 0.28</td>
<td>4.64 ± 0.20</td>
<td>5.68 ± 0.26</td>
</tr>
<tr>
<td>PRONO3</td>
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<td>4.34 ± 0.28</td>
<td>5.69 ± 0.29</td>
</tr>
<tr>
<td>L-[1-13C]phenylalanine, MPE</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>PRO</td>
<td>11</td>
<td>NA</td>
<td>NA</td>
<td>5.44 ± 0.68</td>
</tr>
<tr>
<td>PRONO3</td>
<td>12</td>
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<td>NA</td>
<td>5.06 ± 0.77</td>
</tr>
</tbody>
</table>

Values are means ± SE. MPE, mole percent excess; NA, not available. Data were analyzed by repeated-measures (time × intervention) ANOVA.
rates, or stimulate incorporation of dietary protein-derived amino acids into de novo myofibrillar protein. These data are the first to demonstrate that acute NO$_3^-$ supplementation does not represent an effective nutritional strategy to enhance postprandial protein handling in vivo in humans. As acute dietary interventions to restore vascular function do not appear to increase postprandial muscle protein synthesis rates (13, 37, 41), longer-term intervention studies involving chronic supplementation of NO substrates may be necessary to enhance vascular function and augment the anabolic response to protein ingestion (30, 48, 59). Furthermore, more clinically compromised subpopulations suffering from macro- and/or microvascular complications may present greater benefits from the use of dietary NO$_3^-$ ingestion.

In conclusion, co-ingestion of dietary NO$_3^-$ with a single meal-like amount of protein increases plasma NO$_3^-$ and NO$_2^-$ concentrations but does not modulate protein digestion and amino acid absorption, augment postprandial whole body or skeletal muscle protein synthesis rates, or stimulate incorporation of dietary protein-derived amino acids into de novo muscle protein synthesis in older, type 2 diabetes patients. Therefore, acute dietary NO$_3^-$ supplementation does not represent an effective nutritional strategy to restore or enhance NO metabolism and improve postprandial protein handling in older, type 2 diabetes patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


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