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 5 h 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-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; aging; 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 NO3 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 NO3 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 NO3 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 m/kg², HbA1C = 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 NaNO3 (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 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 NO3 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.

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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/m²</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/m²</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>Plasma insulin (OGTT at t = 120 min), ml/l</td>
<td>16.8 ± 2.4</td>
<td>17.4 ± 1.6</td>
</tr>
<tr>
<td>Plasma insulin (OGTT at t = 120 min), ml/l</td>
<td>45.6 ± 8.0</td>
<td>42.3 ± 7.1</td>
</tr>
<tr>
<td>Hba1c, %</td>
<td>7.1 ± 0.3</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>OGIS, ml/mg·min⁻¹·m⁻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 co-ingestion; PRONO3, protein with NaNO3 co-ingestion; BMI, body mass index; HbA1c, 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.
tube, housed in a gas-phase chemiluminescence NO analyzer (Sievers NOA 280; Analytic). 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-13C6,15N]phenylalanine, and [U-13C6,15N]tyrosine 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 determined using selective 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-tracer 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-(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-13C6,15N]-labeled phenylalanine, respectively, and m/z 466 and 476 for unlabeled and [U-13C6,15N]-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-2H5) 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 L-[1-13C]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 for the presence of the 13C and H2 isotopes. Plasma isotopic enrichment of the t-BDMS derivative of α-[13C]ketosiccaprate (KIC) was measured by GC-MS analysis for use as a surrogate for leucyl-RNA labeling (52).

**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/g wet weight) and then centrifuged at 10,000 g and 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 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. The supernatant 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 used for GC-MS analysis. 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 24 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, 100-200 µm mesh hydrogen 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-butylmethylisilyl-N-methyltrifluoroacetamide-phenylalanine (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 t-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-tracer 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,N-diethylcarbomethyl ester derivatives with ethyl chloroformate for determination of the t-BDMS derivative of α-[13C]ketosiccaprate (KIC) was measured by GC-MS analysis for use as a surrogate for leucyl-RNA labeling (52).

**Calculations.** Whole body amino acid kinetics in non-steady-state conditions were calculated from ingestion of L-[1-13C6]phenylalanine-labeled protein, intravenous infusion of L-[ring-2H5]phenylalanine and L-[ring-3,5-2H2]tyrosine, and arterialized venous blood sampling. Total, exogenous, and endogenous phenylalanine appearance rates (Ra), 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).

\[
\text{total } R_a = \frac{F_v}{E_v(t)} \left[ pV \cdot C(t) \cdot \frac{dE_v}{dt} \right]
\]  

Total phenylalanine Ra was calculated using Eq. 1, where \( F_v \) 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 \( dE_v/dt \) represents the time-dependent change in plasma phenylalanine enrichment derived from the intravenous tracer and \( E_v(t) \) is the mean plasma phenylalanine enrichment from the intravenous tracer between two consecutive time points.

Exogenous phenylalanine \( R_a \) (Eq. 2; Exo \( R_a \)) represents the plasma entry rate of dietary protein-derived phenylalanine. \( E_v(t) \) is the mean plasma phenylalanine enrichment of the oral tracer, \( dE_v/dt \) represents the time-dependent change in plasma phenylalanine enrichment derived from the oral tracer, and \( E_{prot} \) is the enrichment of L-[1-13C6]phenylalanine in the diet protein.

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

\[
\text{pPh}_{\text{plasma}} = \frac{\text{AUC}_{\text{Exo}R_a}}{\text{pPh}_{\text{prot}}} \cdot \text{BW} \cdot 100\%
\]
Phe\textsubscript{plasma} was calculated using Eq. 4. AUC\textsubscript{ExoRa} is the area under the curve (AUC) of Exo Ra, BW is the body weight of the subject (in kg), and Phe\textsubscript{net} is the amount of dietary phenylalanine that is ingested.

Total phenylalanine rate of disappearance (Eq. 5: \( \text{Ra} \text{, in \( \mu \text{mol Phe-kg}^{-1}\text{min}^{-1} \) equals the rate of phenylalanine hydroxylation (the 1st step in phenylalanine oxidation) and utilization for protein synthesis. These parameters are calculated as follows:

\[
\text{total Ra} = \text{total Ra} - pV \cdot \frac{dC}{dt}
\]  

(5)

\[
\text{Phe hydroxylation} = \text{Tyr Ra} \cdot \frac{E_{\text{tyr}(t)}}{E_{\text{phe}(t)}} \cdot \frac{\text{PheRa}}{\left(F_{\text{phe}} + \text{PheRa}\right)}
\]  

(6)

\[
\text{protein synthesis} = \text{total Ra} - \text{Phe hydroxylation}
\]  

(7)

\[
\text{Phe net balance} = \text{protein synthesis} - \text{endogenous Ra}
\]  

(8)

Tyr Ra is the total rate of tyrosine appearance based on the L-[ring-2H5]tyrosine infusion and plasma enrichment. \( E_{\text{tyr}(t)} \) and \( E_{\text{phe}(t)} \) represent the mean plasma L-[ring-2H5]tyrosine and L-[ring-2H5]phenylalanine enrichment between two consecutive time points, respectively. \( F_{\text{phe}} \) is the intravenous infusion rate of L-[ring-2H5]phenylalanine (in \( \mu \text{mol-kg}^{-1}\text{min}^{-1} \)).

Myofibrillar protein fractional synthesis rates (FSR) were calculated using the standard precursor-product equation

\[
\text{FSR} = \frac{\Delta E_{\text{P}}}{E_{\text{precursor}}} \cdot 100\%
\]  

(9)

where \( \Delta E_{\text{P}} \) is the increment in myofibrillar protein-bound L-[ring-2H5]phenylalanine or L-[1-13C]leucine enrichments after an incorporation period (in mole percent excess; MPE). \( E_{\text{precursor}} \) is the average plasma L-[ring-2H5]phenylalanine or \( \alpha \)-[1-13C]KIC enrichment during the tracer incorporation period (in MPE), and \( t \) is the incorporation period (in hours). For basal FSR, muscle biopsies at -120 and 0 min were used; for postprandial FSR, biopsies at 0, 120, and 300 min were used to calculate the early (0–2 h) and late (2–5 h) postprandial muscle protein synthetic response.

**Statistics.** Values are means ± SE. Differences in baseline values were determined using an unpaired, two-tailed Student’s t-test. Incremental AUC (iAUC), peak value, and time to peak were calculated for all plasma time courses, and differences were determined using an unpaired, two-tailed Student’s t-test. Two-way ANOVA with time as the within-group factor and intervention as the between-group factor was used to compare differences over time in plasma glucose, insulin, NO\textsubscript{3} -, NO\textsubscript{2} -, amino acid concentrations and enrichments, whole body phenylalanine \( \text{Rd} \), and FSR. In case of significant interaction between time and intervention, Bonferroni’s post hoc test was applied. Differences between intervention groups in muscle free and protein-bound amino acid enrichments were analyzed with an unpaired, two-tailed Student’s t-test. For all analyses, statistical significance was set at \( P < 0.05 \). All calculations were performed using IBM SPSS Statistics (version 21).

**RESULTS**

**Plasma analyses.** Plasma NO\textsubscript{3} - and NO\textsubscript{2} - concentrations are presented in Fig. 1. Plasma NO\textsubscript{3} - concentrations (Fig. 1A) did not differ at baseline (-210 min) between intervention groups and increased following NaNO\textsubscript{3} ingestion (\( P < 0.001 \)). Throughout the experiment, NO\textsubscript{3} - concentrations were significantly higher in the PRONO3 than the PRO group (\( P < 0.001 \), time \( \times \) intervention interaction \( P < 0.001 \), iAUC \( P < 0.001 \)), reaching peak values of 663 ± 22 and 39 ± 2 \( \mu \text{mol/l} \) in the PRONO3 and PRO groups, respectively (\( P < 0.001 \)). Plasma NO\textsubscript{2} - concentrations (Fig. 1B) increased significantly following NaNO\textsubscript{3} ingestion in the PRONO3 group only (time effect \( P < 0.01 \), group effect \( P < 0.05 \), time \( \times \) 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 \( \times \) 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-2H5]phenylalanine (Fig. 4A) and L-[1-13C]leucine (Fig. 4C) enrichments did not differ between interventions in the basal and postprandial periods. Plasma L-[1-13C]phenylalanine enrichments (Fig. 4B) increased following protein ingestion.
Whole body protein breakdown rates decreased following protein ingestion (P < 0.001). 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 ± 0.001 µmol·kg·h⁻¹; iAUC. Values are means ± SE. Data were analyzed with repeated-measures (time × intervention) ANOVA.

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.

Whole body phenylalanine kinetics. Whole body phenylalanine Rₘ values are presented in Fig. 5. Exogenous phenylalanine Rₘ (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 Rₘ (Fig. 5B) decreased following protein ingestion and did not differ between intervention groups. No differences were detected between interventions in total plasma phenylalanine Rₘ (Fig. 5C) and Rₐ (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 ± 0.001 µmol·kg·h⁻¹; iAUC. Values are means ± SE. Data were analyzed with repeated-measures (time × intervention) ANOVA.

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.
0.004 and 0.021 ± 0.007%/h, respectively, in the early (0–2 h) postprandial phase and 0.032 ± 0.004 and 0.030 ± 0.003%/h, respectively, in the late (2–5 h) postprandial phase, with no differences between groups (time effect \( P < 0.05 \), intervention effect \( P = 0.7 \)). Postabsorptive myofibrillar FSR based on the \( L\)-[1-\( ^{13}C \)]leucine tracer (Fig. 7B), with plasma \( \alpha \)-[1-\( ^{13}C \)]KIC enrichments used as precursor pool, did not differ between groups (\( P = 0.8 \)). After protein ingestion, early and late postprandial muscle protein synthesis rates in PRO and 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-\( ^{2}H_{5} \)]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 \( NO \) 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 (\( NO \))-\( NO \) 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 \( NO_{2}^{-} \) 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 L-[1-13C]phenylalanine-labeled protein with a primed continuous L-[ring-2H5]phenylalanine infusion, we were able to measure protein-derived L-[1-13C]phenylalanine enrichments (Fig. 4) and quantify the Ra of dietary protein-derived amino acids into the circulation (Fig. 5) (8, 45). Plasma L-[1-13C]phenylalanine enrichments increased directly following 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.

Fig. 5. Exogenous rate of appearance (Ra, A), endogenous Ra (B), total Ra (C), and total rate of disappearance (Rd, D) 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.

Fig. 6. Whole body protein metabolism during basal and postprandial periods 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. Whole body protein breakdown rates: time effect \(P < 0.001\); whole body protein synthesis rates: time effect \(P < 0.001\); whole body protein oxidation rates: time effect \(P < 0.001\), basal between-groups \(P = 0.01\), postprandial between-groups \(P = 0.04\); whole body protein net balance: time effect \(P < 0.001\), basal between-groups \(P = 0.02\). *Significant time effect, \(P < 0.01\). #Significant difference between groups, \(P < 0.05\).
protein-derived L-[1-13C]phenylalanine into de novo muscle tissue (Fig. 8). Protein ingestion resulted in a substantial increase in myofibrillar protein-bound L-[1-13C]phenylalanine enrichments at 2 and 5 h, but no additional effect of dietary NO3 co-ingestion was observed.

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 < 0.001, *Significant time effect, P < 0.05.

Table 2. Muscle tissue free enrichments

<table>
<thead>
<tr>
<th></th>
<th>n</th>
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<th>0 h</th>
<th>2 h</th>
<th>5 h</th>
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<tbody>
<tr>
<td>L-[ring-2H5]phenylalanine, MPE</td>
<td>PRO 11</td>
<td>4.37 ± 0.42</td>
<td>5.50 ± 0.38</td>
<td>5.00 ± 0.26</td>
<td>5.91 ± 0.20</td>
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<td></td>
<td>PRONO3 12</td>
<td>4.40 ± 0.44</td>
<td>5.31 ± 0.48</td>
<td>5.14 ± 0.28</td>
<td>5.87 ± 0.33</td>
</tr>
<tr>
<td>L-[1-13C]leucine, MPE</td>
<td>PRO 11</td>
<td>3.51 ± 0.28</td>
<td>4.64 ± 0.20</td>
<td>5.68 ± 0.26</td>
<td>5.74 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>PRONO3 12</td>
<td>3.47 ± 0.23</td>
<td>4.34 ± 0.28</td>
<td>5.69 ± 0.29</td>
<td>5.85 ± 0.21</td>
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<tr>
<td>L-[1-13C]phenylalanine, MPE</td>
<td>PRO 11</td>
<td>NA</td>
<td>NA</td>
<td>5.44 ± 0.68</td>
<td>3.10 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>PRONO3 12</td>
<td>NA</td>
<td>NA</td>
<td>5.06 ± 0.77</td>
<td>3.59 ± 0.23</td>
</tr>
</tbody>
</table>

Values are means ± SE. MPE, mole percent excess; NA, not available. Data were analyzed by repeated-measures (time × intervention) ANOVA.

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.
Dietary NITRATE CO-INGESTION AND MUSCLE PROTEIN SYNTHESIS

E333

rates, or stimulate incorporation of dietary protein-derived amino acids into de novo myofibrillar protein. These data are the first to demonstrate that acute NO3− supplementation does not represent an effective nutritional strategy to restore postprandial protein synthesis rates or stimulate incorporation of dietary protein-derived amino acids into de novo muscle protein synthesis rates in older, type 2 diabetes patients. Therefore, acute dietary NO3− 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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GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


