Protein supplementation elicits greater gains in maximal oxygen uptake capacity and stimulates lean mass accretion during prolonged endurance training: a double-blind randomized controlled trial

Pim Knuiman,[1] Luc JC van Loon,[2] Jeroen Wouters,[3] Maria Hopman,[1,4] and Marco Mensink[1]

[1]Division of Human Nutrition, Wageningen University and Research, Wageningen, Netherlands; [2]Department of Human Biology, Faculty of Health, Medicine, and Life Sciences, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, Netherlands; [3]Sportcentre Papendal, Centre for Sporting Excellence and Education, Arnhem, Netherlands; and [4]Department of Physiology, Radboud University Medical Centre, Nijmegen, Netherlands

ABSTRACT

Background: Endurance training induces numerous cardio vascular and skeletal muscle adaptations, thereby increasing maximal oxygen uptake capacity (VO_{2max}). Whether protein supplementation enhances these adaptations remains unclear.

Objective: The present study was designed to determine the impact of protein supplementation on changes in VO_{2max} during prolonged endurance training.

Methods: We used a double-blind randomized controlled trial with repeated measures among 44 recreationally active, young males. Subjects performed 3 endurance training sessions per week for 10 wk. Supplements were provided immediately after each exercise session and daily before sleep, providing either protein (PRO group; $n = 19$; 21.5 ± 0.4 y) or an isocaloric amount of carbohydrate as control (CON group; $n = 21$; 22.5 ± 0.5 y). The VO_{2max}, simulated 10-km time trial performance, and body composition (dual-energy X-ray absorptiometry) were measured before and after 5 and 10 wk of endurance training. Fasting skeletal muscle tissue samples were taken before and after 5 and 10 wk to measure skeletal muscle oxidative capacity, and fasting blood samples were taken every 2 wk to measure hematological factors.

Results: VO_{2max} increased to a greater extent in the PRO group than in the CON group after 5 wk (from 49.9 ± 0.8 to 54.9 ± 1.1 vs 50.8 ± 0.9 to 53.0 ± 1.1 mL · kg^{-1} · min^{-1}; $P < 0.05$) and 10 wk (from 49.9 ± 0.8 to 55.4 ± 0.9 vs 50.8 ± 0.9 to 53.9 ± 1.2 mL · kg^{-1} · min^{-1}; $P < 0.05$). Lean body mass increased in the PRO group whereas lean body mass in the CON group remained stable during the first 5 wk (1.5 ± 0.2 vs 0.1 ± 0.3 kg; $P < 0.05$) and after 10 wk (1.5 ± 0.3 vs 0.4 ± 0.3 kg; $P < 0.05$). Throughout the intervention, fat mass reduced significantly in the PRO group and there were no changes in the CON group after 5 wk (−0.6 ± 0.2 vs −0.1 ± 0.2 kg; $P > 0.05$) and 10 wk (−1.2 ± 0.4 vs −0.2 ± 0.2 kg; $P < 0.05$).

Conclusions: Protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion but did not improve skeletal muscle oxidative capacity and endurance performance during 10 wk of endurance training in healthy, young males. This trial was registered at clinicaltrials.gov as NCT03462381. *Am J Clin Nutr* 2019;110:508–518.

Keywords: protein supplementation, endurance training, maximal oxygen uptake capacity, skeletal muscle oxidative capacity, body composition

Introduction

Endurance training represents an effective strategy to increase maximal oxygen uptake capacity (VO_{2max}) (1, 2). An increase in VO_{2max} is independently associated with a reduction in all-cause mortality, which emphasizes the important clinical benefits of endurance training (3). Increases in VO_{2max} as a result of endurance training can be attributed to adaptive responses of several organ systems involved in the oxygen transport and utilization chain, from lungs, heart, and vasculature to the mitochondria in muscle tissue (4–7). The impact of different endurance training regimes on both cardiovascular (8–11) and skeletal muscle

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Data availability for editors: The authors confirm to make the data used in the manuscript, code book, and analytic code available to editors upon request either before or after publication.

Address correspondence to PK (e-mail: pim.knuiman@wur.nl).

Abbreviations used: CON, control group (carbohydrate-supplemented); CS, citrate synthase; CytC, cytochrome C oxidase; DXA, dual-energy X-ray absorptiometry; HR, heart rate; IPAQ, International Physical Activity Questionnaire; MET, metabolic equivalent of task; PRO, protein group (protein-supplemented); RPE, rate of perceived exertion; VO_{2max}, maximal oxygen uptake capacity.

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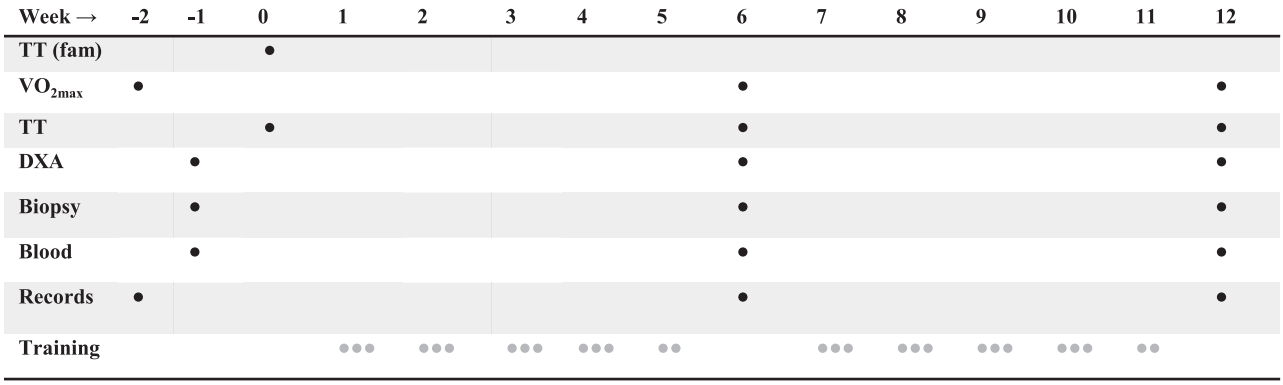

FIGURE 1 Forty subjects completed 10 wk of exercise training while consuming either 29 g calcium casein protein or 29 g carbohydrates postexercise and daily before sleep. Measurements and biopsies were performed at Pre (week 0), Mid (week 6), and End (week 12), whereas blood samples were taken at weeks 0, 2, 4, 6, 8, 10 and 12. TT (fam): 10-km time trial familiarization, TT: 10-km time trial, VO_{2max}: maximal oxidative capacity exercise test. Black dots: measurement points, gray dots: exercise training sessions. TT fam and TT baseline were performed within the same week with 3–4 d between time trials.

(5, 12–15) adaptations have been thoroughly investigated. In contrast, only a few studies have determined the impact of protein supplementation on the adaptive response to endurance training, with conflicting findings (16–18). Additionally, these studies were either underpowered (17) or not double blinded (18), or included exercise training sessions that were not fully monitored by investigators (16), which makes it difficult to draw conclusions about the potential effectiveness of protein supplementation for endurance training adaptation. Clearly there is a strong need for well-powered double-blind randomized controlled trials to establish whether protein supplementation impacts the adaptive response to endurance training.

Therefore, we designed a double-blind randomized controlled trial with a supplementation strategy that theoretically should be effective based upon previous postexercise and before-sleep protein fractional synthetic rate measurements (19, 20). Our main purpose was to determine the impact of protein supplementation on changes in VO_{2max} following 10 wk of endurance training. Secondarily, we were also interested in skeletal muscle oxidative capacity, endurance performance, hematological factors, and body composition. To gain insight into the timespan of adaptations we assessed all outcome measures after both 5 and 10 wk of training. We hypothesized that protein supplementation facilitates the adaptive response to endurance training.

Methods

Subjects

Forty-four young healthy males volunteered and gave full written informed consent to participate in a 10-wk endurance training program, with or without additional protein supplementation. Primary inclusion criteria were nonsmoker, free of injury, and not using any medication or nutritional supplements. Additional exclusion criteria that would preclude successful participation in the training study included (diagnosed) lactose intolerance and/or dairy protein allergy, cardiorespiratory-related illness, and musculoskeletal-related injuries that would impede endurance training sessions. All subjects were physically active, performing sports on a noncompetitive basis between 1 and 4 h/wk. None of the participants had a history of participating in any structured endurance training programs to improve performance within the past 2 y. The present study was carried out in accordance with the guidelines for human research of the Medical Ethical Committee of Wageningen University. The Medical Ethical Committee of Wageningen University approved all study procedures and complied with the guidelines set by the Declaration of Helsinki of 1975 as revised in 1983. This trial was registered at clinicaltrials.gov as NCT03462381.

Experimental design

Subjects completed a progressive endurance training protocol with 3 exercise sessions per week for a total of 10 wk and consumed a postexercise and presleep drink providing either protein or an isocaloric amount of carbohydrate. The total study duration, including a series of midterm and end measurements, was ∼12 wk. After inclusion, subjects were randomly assigned to either a protein-supplemented (PRO) or carbohydrate-supplemented control (CON) group. Before (week 0), during (week 6), and after (week 12) the exercise training program, anthropometric measurements (height, body mass, and waist circumference), VO_{2max} ramp tests, simulated 10-km time trials, and dual-energy X-ray absorptiometry (DXA) scans were carried out. In addition, muscle biopsy specimens, dietary intake records, and physical activity records were collected (**Figure 1**). Fasting blood samples were taken at week 0 (pre); 2, 4, and 6 (mid); and 8, 10, and 12 (end). The subjects were instructed to maintain their normal dietary habits and physical activity patterns throughout the intervention period. A standardized meal was provided the evening before each test day (standard deep-frozen meal and ice cream dessert; 43.80 kJ/kg BW; 15 Energy% protein, 30 Energy% fat, and 55 Energy% carbohydrate; Roerbaksensatie, Iglo). Subjects refrained from continuous physical activity for at least 72 h before testing. On the different test days, subjects arrived at the laboratory after an overnight fast.

Endurance training program

Training intervention was divided into 2 blocks of 5 wk with 1 wk (week 6) in between the blocks to perform the midterm

TABLE 1 Nutritional composition of the intervention drinks (250 mL)

Energy and nutrient	Control beverage (CON)	Protein beverage (PRO)
Energy, kcal	~129	~127
Protein (casein), g	0.6	28.7
Fat, g	2.4	0.3
Carbohydrates (maltodextrin and sucrose), g	26.3	2.7

measurements, and a final week (week 12) to perform the end measurements. During the first 4 wk subjects participated in 3 endurance exercise sessions/wk alternated with 1 d of rest between each session. On week 5 subjects performed 2 endurance exercise sessions. This was repeated in the second part of the study (weeks 7–11), see Figure 1. In total, subjects performed 28 endurance training sessions. After a 10-min warmup on a cycle ergometer, the endurance training session consisted of 60-min continuous cycling. All training sessions were conducted under the supervision of a researcher using indoor, mechanically braked spinning bikes (Body Bike Smart, Body Bike International) with participants free to adjust resistance and cadence as desired. Heart rate (HR) for each session was recorded (Polar Electro), HR and rate of perceived exertion (RPE) were taken at the start and every 5 min thereafter by the supervisor. Music and verbal motivation were provided during training sessions, which were conducted under ambient conditions at sea level at thermal-neutral conditions (21°C, 40% relative humidity). Each endurance training session ended with a 10-min cooling down period on the same cycle ergometer. Exercise intensity was determined using the Karvonen formula (21):

$$Intensity = HRreserve \cdot 0.85 + HRrest \quad (1)$$

Where HRreserve is the calculated difference between HRmax (determined during VO_{2max} ramp test) and HRrest. According to the American College of Sports Medicine, the selected exercise intensity can be considered "vigorous" (22).

Protein and control supplementation

Subjects consumed a 250-mL beverage containing either 29 casein protein or an isocaloric amount of carbohydrate immediately after cessation of each exercise session (3 per wk) and every day before sleep (7 per wk). In addition, all subjects received 2 slices of gingerbread directly after every training session (total energy 280 Kcal; 63.2 g carbohydrates; 1.4 g fat; 2.4 g protein). An overview of the energy and macronutrient composition of the beverages can be found in **Table 1**. Nutritional content of the supplements was analyzed in duplicate by an independent laboratory (NutrilabRijswijk) and reported analogous nutritional values as given by the producer. The protein and control beverages were masked for taste and smell by adding several additives. In addition, beverages were masked for color and produced in white nontransparent containers. Assignment to the PRO or CON group was done using block randomized assignment (group 1, $n = 24$; group 2, $n = 20$) by an independent researcher not involved in the study. Study drink boxes/beverages were sequentially numbered according to subject number.

Assessment of blinding success

Determination of blinding success was done using the blinding index method described by James et al. (23). Following the intervention subjects were asked what treatment they thought they have had and could choose between "carbohydrate/protein" and "I do not know." Subsequently, weights were given for subjects to guess (0 for correct guess; 0.5 for incorrect guess; and 1 for "I do not know").

Habitual dietary intake and physical activity

Over the course of the intervention period, subjects maintained their habitual dietary intake and physical activity pattern. Subjects recorded 3-d (days were randomized, but in general 2 weekdays and 1 weekend day) weighted dietary intake records to assess potential changes in daily food intake that might have occurred over the course of the intervention period (week 5), before the onset of the intervention (week 0), and at week 10 of the intervention. Dietary intake records were analyzed using Compl-eat (Human Nutrition). Habitual physical activity and supplemental exercise were assessed using the extended version of the International Physical Activity Questionnaire (IPAQ). The extended IPAQ is a self-administered 7-d physical activity recall questionnaire. Calculation and quantification of physical activity scores were determined according to the guidelines for data processing and analysis of the IPAQ, as described elsewhere (24).

Body composition

The DXA measurements were carried out after an overnight fast using a Lunar Prodigy Advanced DXA scanner (GE Health Care). Each morning on the different test days, a quality assurance test was performed to ensure system suitability and precision of the scanner. Whole-body scans were performed according to the manufacturer's protocol and identical scan protocols were used for all subjects. Subsequently, different regions for fat mass and lean mass were assessed. Anthropometrics were assessed using standardized procedures, body weight by digital scale to within 100 g, height by stadiometer to within 0.5 cm, and waist circumference by tape measure to within 0.5 cm (SECA Medical Measuring Systems and Scales).

VO_{2max}

A ramped VO_{2max} test was performed at baseline (−2 wk), midterm, and end (2–3 d after the last training sessions) between 0900 and 1700. Ninety minutes before each test, subjects consumed a standardized meal consisting of an energy bar (3.7 g fat, 29.2 g carbohydrates, 2 g protein, 158.1 Kcal), an apple (Granny Smith), and 500 ml water. Following a 30-min rest, subjects performed a ramped VO_{2max} test on an electrically braked cycle ergometer (Lode Excalibur). After a 5-min warmup at 50 W, the subjects started cycling at 100 W. Workload was progressively increased by 20 $W \cdot min^{-1}$ until the subject reached volitional exhaustion. The VO_{2max} test was considered to be valid when 2 out of 3 criteria were met: *1*) levelling of VO_2 with increasing workload; *2*) heart rate within 10 beats of the theoretically estimated maximum (220 − age); and *3*) respiratory exchange ratio of ≥1.15. Oxygen consumption (VO_2) was

measured through breath-by-breath sampling with an Oxycon Pro (Jaeger) to define VO_{2max}. Subjects were asked to maintain a cadence between 80 and 100 rounds·min^{-1}.

Endurance exercise performance (simulated 10-km time trial)

Familiarization was performed in the week after the start of the first experimental day (anthropometrics, DXA, blood, and biopsy). Three or 4 d thereafter another time trial was conducted (baseline measurement) and this was repeated at midterm and end. Subjects performed a simulated ∼10-km cycling time trial. The data from the baseline VO_{2max} test was used for the amount of work to be performed and calculated as follows: total amount of work (J) = 0.85 × Wmax × 900 (s) (25). The ergometer was set in linear-mode so that 85% Wmax was achieved when subjects cycled at their preferred pedaling rate of 85 ± 7 rpm, as determined during familiarization. Subjects received no verbal or physiological feedback during the time trial, and were only aware of the absolute (kJ) and relative (%) amount of work performed. The RPE were assessed after each 30-min submaximal exercise test and after the time trial using the Borg 6–20 scale (26). All testing was performed under standardized conditions (21°C, 40% relative humidity) at the same time of day and on the same day in the week.

Blood sampling and analysis

Resting blood samples were collected after an overnight fast at weeks 0, 2, 4, 6, 8, 10, and 12 in EDTA-coated evacuated tubes (BD Biosciences) by venipuncture. Whole blood was analyzed for red blood cells, hemoglobin concentration, and hematocrit.

Muscle biopsies

Muscle biopsy specimens were taken after an overnight fast 3–4 d prior to the start of the first training session (pre), 5–7 d after training sessions 14 (midterm) and 28 (end). Muscle biopsy specimens were taken as described by Bergstrom (27). Biopsies were performed under local anesthesia (2–3 mL 2% adrenaline) using a 5-mm Bergstrom needle modified with suction. Biopsy specimens were taken from the vastus lateralis of the same leg, with separate incisions (∼1–1.5 cm apart) and from distal to proximal direction. Muscle biopsy specimens were immediately frozen (within 5–10 s) in liquid nitrogen and stored at −80°C for subsequent biochemical analysis, after being freed from visible fat, blood, and connective tissue.

Oxidative enzymes

Wet muscle was used for determining indices of skeletal muscle oxidative capacity. In approximately 30 mg muscle tissue (maximal) citrate synthase (CS) and cytochrome C oxidase (CytC) activity were measured according to methods published previously (28). Both enzyme activities are expressed as micromoles of product (citrate and reduced cytochrome C for CS and CytC, respectively) generated per gram of wet muscle tissue per minute during the assay (μmol·g^{-1}·min^{-1}).

Statistics

Power calculation.

Sample size (n) was calculated with 90% power and type I error probability of 0.05 based on the primary outcome of VO_{2max} increase. An expected and/or relevant difference in VO_{2max} seen with endurance training is 0.32 ± 0.30 (L·min^{-1}) (29). To demonstrate a statistically significant greater gain in VO_{2max} by a nutritional supplement, 16 participants/group have been shown to be sufficient (18). Considering a dropout rate of 20–30%, the final number of the participants included was 22/group.

General statistics.

An independent t -test was carried out to verify that groups were similar at baseline. Data were assessed for normality with the use of a Shapiro-Wilk test, and any nonnormal data (time trial results, CS, and CytC) were corrected with the use of transformation, with the type of transformation based on the nature of the skewedness of the data. Repeated measures of ANOVA (2-way mixed ANOVA) was used to determine statistical significance for the dependent variables over time. The ANOVA model for the dependent variables with 3 testing time points was described as $S_{40}\times T_3\times G_2$ such that (S; number of subjects) are crossed with testing time [T; 3 testing times: Pre (week 0), Mid (week 6), and End (week 12)] and group (G; CON and PRO), and for blood sample analysis $S_{40}\times T_7\times G_2$. Where 2-way mixed ANOVA revealed significant interaction, a Tukey's post hoc test was conducted for multiple comparisons to further analyze within-group effects and unpaired t-tests to compare between groups at specific time points. In case 2-way mixed ANOVA revealed no significant interaction but significant main effects of time and/or group, pairwise comparisons with Tukey's post hoc correction were done. Data management and statistical analysis were carried out using SPSS software version 23 (SPSS Inc.). Statistical significance was declared when $P < 0.05$. Figures were prepared using GraphPad Prism 8.01 for Windows. All data are expressed as mean ± SEM.

Results

Baseline characteristics

Four subjects dropped out during the study, one because of relocation, one because of a hamstring injury, one because of a knee injury and one because of appendicitis. Final analysis was performed on the 40 subjects who completed the training program (CON: $n = 21$ vs. PRO: $n = 19$) (**Figure 2**). Baseline characteristics of both groups prior to the endurance training are summarized in **Table 2**. There were no differences at baseline between the CON and PRO groups in any of the variables of interest.

Endurance training adherence and supplement intake

All training sessions were performed between 0900 and 2100 with no differences between groups. On average, subjects attended 98 ± 0.3% and 98.7 ± 0.3% of the sessions in the CON and PRO groups, respectively, with no differences between groups. There was no significant time-by-treatment interaction

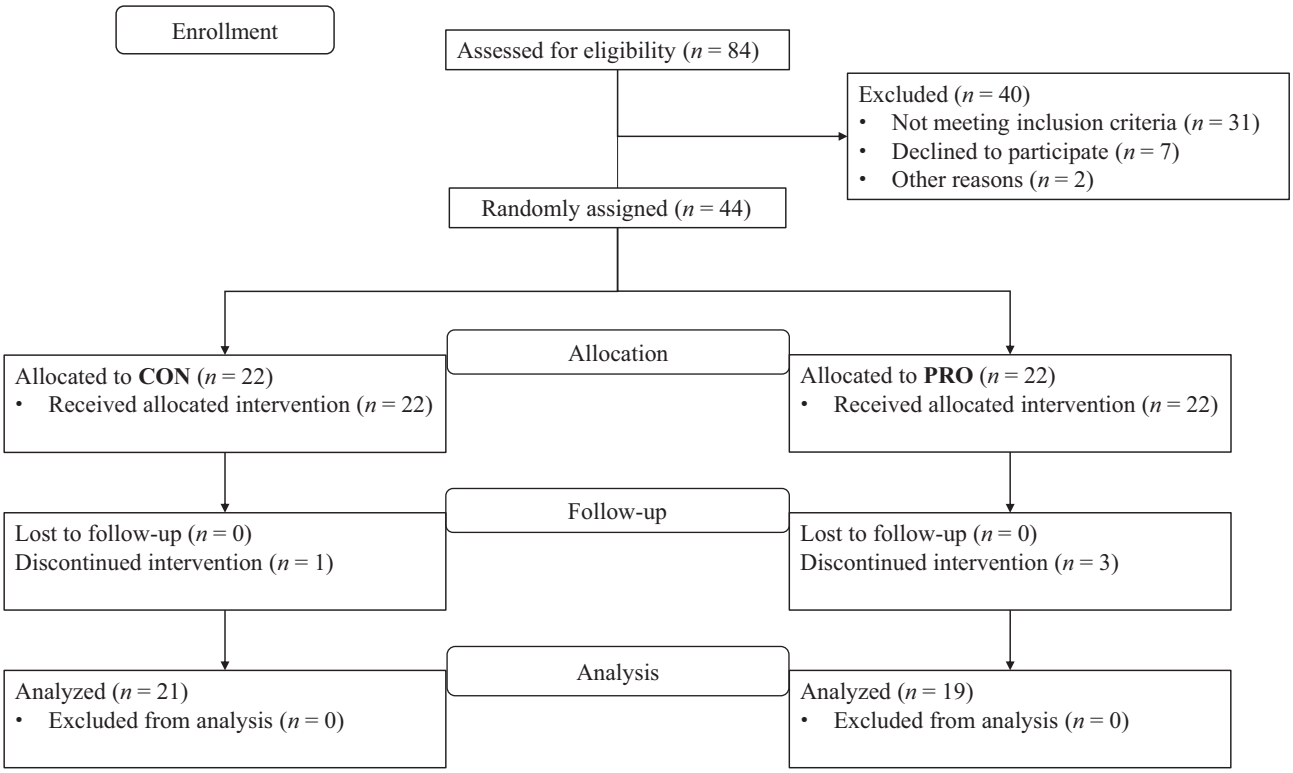

FIGURE 2 Subject recruitment and flow through the protocol. CON (carbohydrate supplementation, $n = 21$); PRO (protein supplementation, $n = 19$).

for exercise intensity as measured by HR per min ($P = 0.37$) or RPE ($P = 0.78$) over the course of the training intervention. The postexercise drinks were consumed under supervision, resulting in a 100% compliance. The consumption of the presleep drinks had an overall compliance of 98.3 ± 0.3%, with no differences between groups. Blinding success was based on the upper bound of the CI of the blinding index. The CI was <0.5, indicating there was insufficient evidence for unblinding (**Table 3**).

Habitual dietary intake and physical activity level

Statistical analysis revealed no differences in energy intake between the groups and/or over time (**Table 4**). As a result of supplementation, carbohydrate intake increased significantly on training days, but not on nontraining days in the CON group. Meanwhile, carbohydrate intake remained stable in the PRO group. Protein supplementation significantly increased protein intake on training days (baseline: 1.3 ± 0.1; midterm: 1.8 ± 0.1; end: 1.8 ± 0.1 $g \cdot kg^{-1} \cdot d^{-1}$) and nontraining days (baseline: 1.3 ± 0.1; midterm: 1.5 ± 0.1; end: 1.7 ± 0.1 $g \cdot kg^{-1} \cdot d^{-1}$) in the PRO group, while protein intake remained stable in the CON group on training days (baseline: 1.3 ± 0.1; midterm: 1.2 ± 0.1; end: 1.3 ± 0.1 $g \cdot kg^{-1} \cdot d^{-1}$) and nontraining days (baseline: 1.3 ± 0.1; midterm: 1.2 ± 0.1; end: 1.3 ± 0.1 $g \cdot kg^{-1} \cdot d^{-1}$). In addition to diet, habitual physical activity/exercise was also monitored. There were no differences in metabolic equivalent of task (MET) per minute per week (excluding exercise sessions) between the groups at baseline, midterm, and end. Habitual physical activity tended to decrease after the onset of the exercise intervention in both the CON (baseline: 2415 ± 248; midterm: 1982 ± 196; end: 2243 ± 248 $MET \cdot min^{-1} \cdot wk^{-1}$) and PRO group (baseline: 2532 ± 275; midterm: 2169 ± 246; end: 2233 ± 305 $MET \cdot min^{-1} \cdot wk^{-1}$).

TABLE 2 Baseline characteristics[1]

Characteristic	CON ($n = 21$)	PRO ($n = 19$)
Age, y	22.5 ± 0.5	21.5 ± 0.4
Body mass, kg	77.2 ± 1.6	76.3 ± 1.3
Height, m	1.85 ± 0.0	1.85 ± 0.0
BMI, kg/m^2	22.4 ± 0.3	22.3 ± 0.4
VO_{2max}, $mL \cdot kg^{-1} \cdot min^{-1}$	50.8 ± 0.9	49.9 ± 0.8

[1] Values are means ± SEM. No significant differences were observed between groups. CON, carbohydrate supplemented; PRO, protein supplemented; VO_{2max}, maximal oxygen uptake capacity.

VO_{2max}

Statistical analysis revealed a significant time-by-treatment interaction for VO_{2max} ($P = 0.033$). The VO_{2max} increased to a greater extent in the PRO than in the CON group after 5 wk (from 49.9 ± 0.8 to 54.9 ± 1.1 vs. 50.8 ± 0.9 to 53.0 ± 1.1 $mL \cdot kg^{-1} \cdot min^{-1}$; $P = 0.017$) and after 10 wk (from 49.9 ± 0.8 to 55.4 ± 0.9 vs. 50.8 ± 0.9 to 53.9 ± 1.2 $mL \cdot kg^{-1} \cdot min^{-1}$; $P = 0.045$) (**Figure 3**). Changes in absolute VO_{2max} and

TABLE 3 Assessment for success of blinding[1]

Guess	CON	PRO	Do not know	Total	BI	95% CI
CON	7 (17)	6 (15)	8 (20)	21 (52)	0.5	0.32, 0.72
PRO	3 (8)	10 (25)	6 (15)	19 (48)	0.4	0.17, 0.62
Total	10 (25)	16 (40)	14 (35)	40 (100)	—	—

[1]Values presented as number of guesses (%), unless otherwise indicated. Determination of blinding success was done using the blinding index method described by James et al. (23). BI, binding index; CON, carbohydrate supplemented; PRO, protein supplemented.

absolute and relative maximal aerobic power (W) were all in line with the changes in VO_{2max} and are displayed in **Table 5**.

10-km simulated time trial performance

As a result of endurance training, 10-km time trial performance improved in both groups ($P < 0.0001$). However, protein supplementation did not enhance the exercise-induced improvements in performance (time-by-treatment interaction $P = 0.64$) (**Figure 4**).

Skeletal muscle mitochondrial enzyme activities

Endurance training increased maximal CS activity in the CON and PRO groups after 5 (21.7 ± 1.3 to 28.7 ± 1.1 vs. 23.4 ± 1.4 to 31.9 ± 1.1 umol·g^{-1}·min^{-1}; $P < 0.05$) and 10 wk (21.7 ± 1.3 to 29.8 ± 1.1 vs. 23.4 ± to 33.9 ± 1.2 umol·g^{-1}·min^{-1}; $P < 0.05$) of training. However, the increases in CS were not statistically different between groups at midterm ($P = 0.21$) and end ($P = 0.11$) (**Figure 5**). There were no differences in changes between the CON and PRO group in maximal CytC activity after 5 (24.8 ± 0.5 to 24.6 ± 0.4 vs. 25.2 ± 0.7 to 24.0 ± 0.7 μmol·g^{-1}·min^{-1}; $P > 0.05$) and 10 wk (24.8 ± 0.5 to 23.9 ± 0.5 vs. 25.2 ± 0.7 to 22.9 ± 0.7 μmol·g^{-1}·min^{-1}; $P > 0.05$) of training (Figure 5).

Hematological factors

Compared with baseline, endurance training caused significant reductions in erythrocytes, hemoglobin, and hematocrit throughout the training period, whereas all 3 factors returned to baseline levels after 10 wk of training. There was no time-by-treatment interaction for any of the hematological factors (**Figure 6**).

Body composition

There was time-by-treatment interaction ($P = 0.049$) for total body mass. Compared to baseline, body mass increased in the PRO group after 5 wk of training, from 76.3 ± 1.3 to 77.2 ± 1.4 kg ($P = 0.001$), but returned to 76.5 ± 1.3 kg ($P = 0.73$) after 10 wk of training. Body mass in the CON group remained stable over the course of the intervention (baseline: 77.2 ± 1.6; midterm: 77.2 ± 1.6; end: 77.4 ± 1.6 kg) ($P = 0.82$). A significant time-by-treatment interaction ($P = 0.001$) for whole-body lean mass was observed (**Table 6**). Lean body mass increased in the PRO group, whereas in the CON group it remained unchanged the first 5 wk (1.5 ± 0.2 vs. 0.1 ± 0.3 kg; $P < 0.0001$) and after 10 wk (1.5 ± 0.3 vs. 0.4 ± 0.3 kg; $P = 0.006$) (**Figure 7**). Furthermore, there was a significant time-by-treatment interaction ($P = 0.015$) for fat mass. The decrease in fat mass between PRO and CON was statistically not different at week 5 (−0.6 ± 0.2 vs. −0.1 ± 0.2 kg; $P = 0.089$) but reached statistical significance at

TABLE 4 Energy intake and macronutrient composition of the diet before, during, and after 10 wk of training in healthy, young men who did receive carbohydrate or protein supplementation[1]

	CON group ($n = 21$)			PRO group ($n = 19$)			P		
	Pre	Mid	End	Pre	Mid	End	Training	Treatment	Interaction
Total energy, kcal/d	2624 ± 96	2620 ± 99	2787 ± 98	2682 ± 103	2558 ± 106	2718 ± 105	NS	NS	NS
Fat, % of energy	34 ± 0.8	35 ± 1.1	34 ± 1.1	38 ± 0.9	34 ± 1.2	35 ± 1.3	NS	NS	NS
Alcohol, % of energy	2.4 ± 0.6	4.2 ± 0.9	5.1 ± 0.9	2.6 ± 0.7	3.1 ± 1.0	3.5 ± 0.9	NS	NS	NS
Carbohydrate, % of energy	47 ± 0.9	46 ± 1.0	47 ± 1.2	43 ± 1.1	47 ± 1.1	46 ± 1.3	NS	NS	0.05
Carbohydrate, g/d	298 ± 12	295 ± 14	314 ± 12	282 ± 12	295 ± 15	299 ± 13	NS	NS	NS
Carbohydrate, g · kg^{-1} · d^{-1}	3.9 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	NS	NS	NS
Carbohydrate intake including supplement, g · kg^{-1} · d^{-1} on nontraining days	3.9 ± 0.2	4.1 ± 0.2	4.4 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	0.02	NS	NS
Carbohydrate intake including supplement, g · kg^{-1} · d^{-1} on training days	3.9 ± 0.2	4.5 ± 0.2*	4.7 ± 0.2*	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	<0.01	<0.01	0.05
Protein, % of energy	15 ± 0.5	15 ± 0.7	16 ± 0.7	15 ± 0.5	15 ± 0.7	16 ± 0.7	NS	NS	NS
Protein intake, g/d	98 ± 3.7	92 ± 4.1	100 ± 4.3	97 ± 4.0	92 ± 4.3	101 ± 4.6	0.03	NS	NS
Protein intake, g · kg^{-1} · d^{-1}	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	0.02	NS	NS
Protein intake, including supplement, g · kg^{-1} · d^{-1} on nontraining days	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1*	1.7 ± 0.1*†	<0.01	<0.01	<0.01
Protein intake, including supplement, g · kg^{-1} · d^{-1} on training days	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.8 ± 0.1*	1.9 ± 0.1*†	<0.01	<0.01	<0.01

[1]Values are means ± SEM. *Significantly different compared with Pre, $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; NS, $P \geq 0.05$; PRO, protein-supplemented.

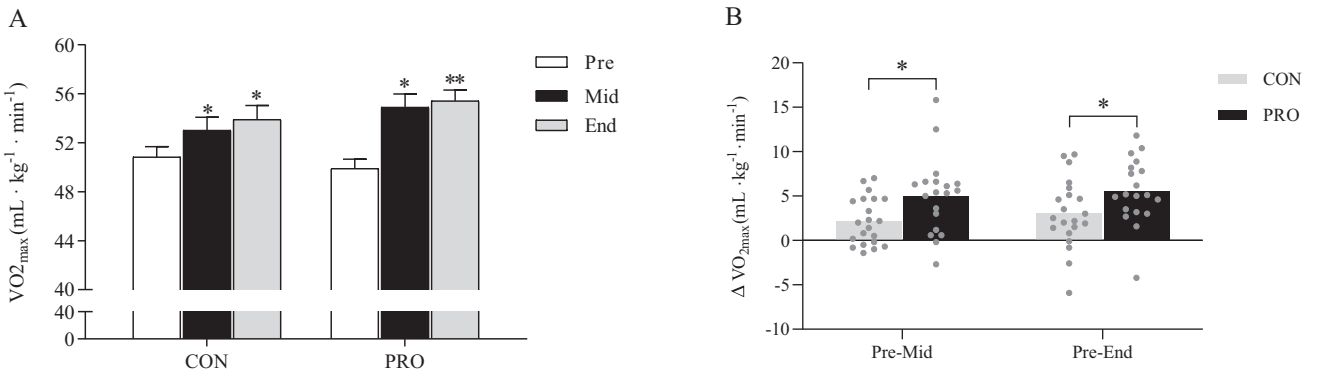

FIGURE 3 (A) Effect of 10 wk of endurance training on maximal aerobic capacity. VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$) during 10 wk of endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Data were analyzed using 2-way mixed ANOVA for time-by-treatment interaction, $P = 0.033$. Tukey's post hoc test was conducted for multiple comparisons within each group. * $P < 0.05$, significant main effect of time compared with Pre. ** $P < 0.0001$, significant main effect of time compared with Pre. (B) Change in maximal aerobic capacity during 10 wk of endurance training. Delta change in VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$) from Pre-Mid and Pre-End during prolonged endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as mean group difference. Gray dots indicate individual subject responses. Data were first analyzed using 2-way mixed ANOVA to detect time-by-treatment interaction, $P = 0.033$. Unpaired *t*-tests were used to compare delta changes between groups from Pre-Mid and Pre-End. * $P < 0.05$ significantly different compared with CON. CON, carbohydrate supplemented; PRO, protein supplemented.

week 10 (−1.2 ± 0.4 vs. −0.2 ± 0.2 kg; $P = 0.021$) (Figure 7). Bone mineral density remained unchanged throughout the intervention in both groups.

Discussion

Ten weeks of endurance training increased VO_{2max}, skeletal muscle oxidative capacity, and 10-km time trial performance. Adding protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion, while promoting fat mass loss. However, protein supplementation did not improve skeletal muscle oxidative capacity and 10-km time trial performance. Our findings confirm our hypothesis that protein supplementation facilitates the adaptive response during prolonged endurance training in healthy, young males.

It is well known that endurance training increases VO_{2max} (1). Our endurance training protocol substantially increased VO_{2max} after 5 and 10 wk, which is in line with previous reports following 5–10 wk of endurance training in healthy, young males (30–32). However, after controlling for the supplementation group, participants in the PRO group showed a greater gain in VO_{2max} than the CON group. The mechanism by which protein supplementation increased VO_{2max} is most likely explained by adaptive responses in both the cardiovascular and musculoskeletal system. Interestingly, in the PRO group, the increase in VO_{2max} during 5 wk of training was accompanied by a substantial gain in lean mass, whereas lean mass remained unchanged in the CON group. In younger healthy individuals, lean mass has been well associated with VO_{2max} (33), as was the case in our study ($r^2 = 0.42$; $P < 0.0001$). Yet, the strongest correlation was found between leg lean mass and VO_{2max} at week 5 ($r^2 = 0.59$; $P < 0.0001$). Changes in leg lean mass correlated mildly but significant with changes in VO_{2max} after 5 wk of training ($r^2 = 0.20$; $P = 0.003$). The greater gain in VO_{2max} we found may also relate to changes in skeletal muscle oxidative capacity, such as mitochondrial density and/or function (34–38). Therefore, we measured CS and CytC activity on a skeletal muscle tissue level as a proxy for mitochondrial content and function (34, 39). The increase in CS activity in skeletal muscle tissue tended to be greater in the PRO than in the CON group, but this difference did not reach statistical significance. Because of the biological individual variation in CS it was not fully unexpected that the difference in CS activity did not reach statistical significance between groups (39). Endurance training slightly decreased maximal CytC activity in the PRO group. This could be explained by the shorter half-life of CytC compared to CS (40). Specifically, muscle biopsies were taken 8 d following the last exercise training session, and considering the relatively short half-life of CytC it could be argued that its activity dropped to pretraining levels and that we may have missed peak levels of maximal activity of CytC throughout the training period (40, 41).

TABLE 5 Absolute VO_{2max} and maximal aerobic power[1]

	CON group ($n = 21$)			PRO group ($n = 19$)			*P*		
	Pre	Mid	End	Pre	Mid	End	Training	Treatment	Interaction
VO_{2max}, L/min	3.87 ± 0.1	4.05 ± 0.1*	4.14 ± 0.1*	3.79 ± 0.1	4.21 ± 0.1*	4.24 ± 0.1*	<0.0001	NS	0.008
Max aerobic power, W/kg	335 ± 6.9	366 ± 6.3	372 ± 6.7	324 ± 7.0	362 ± 7.5	377 ± 7.9	<0.0001	NS	0.053
Max aerobic power, W	4.4 ± 0.1	4.8 ± 0.1*	4.9 ± 0.1*	4.3 ± 0.1	4.7 ± 0.1*	4.9 ± 0.1*†	<0.0001	NS	0.016

[1] Values are means ± SEM. *Significantly different compared with Pre, $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; Max, maximal; NS, $P \geq 0.05$; PRO, protein-supplemented.

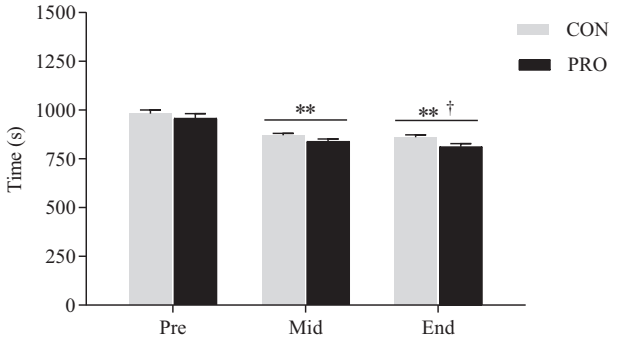

FIGURE 4 Endurance performance (seconds) as measured by a simulated 10-km time trial performance bicycle test during 10 wk of endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Data were analyzed using 2-way mixed ANOVA for time-by-treatment interaction, $P = 0.642$. Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. $^{**}P < 0.0001$ significant main effect of time compared with Pre; $^{\dagger}P < 0.05$ significant main effect of time compared with Mid. CON, carbohydrate supplemented; PRO, protein supplemented.

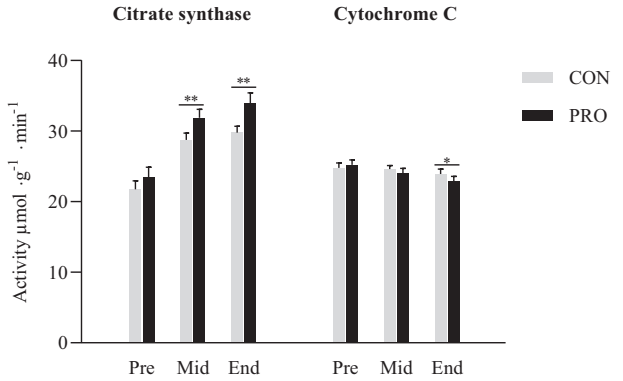

FIGURE 5 Skeletal muscle citrate synthase and cytochrome C oxidase activity during 10 wk of endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Enzyme activities are expressed as micromoles of product (citrate and reduced cytochrome C) generated per gram of wet tissue per min during the assay ($\mu mol \cdot g^{-1} \cdot min^{-1}$). Data were analyzed using 2-way mixed ANOVA for time-by-treatment interaction for citrate synthase, $P = 0.179$, and cytochrome C, $P = 0.292$. Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. $^{*}P < 0.05$ significant main effect of time compared with Pre; $^{**}P < 0.0001$ significant main effect of time compared with Pre. CON, carbohydrate supplemented; PRO, protein supplemented.

While a discussion on the main determinants of improvements in VO_{2max} falls outside the scope of this investigation, recent evidence suggests that hematological adaptations may play a key role herein (29, 42). Yet, we found similar changes in concentrations of erythrocytes, hemoglobin, and hematocrit between the PRO and CON groups, suggesting that the differences in VO_{2max} between the groups cannot be explained by changes in the oxygen-carrying capacity of blood. Adaptations in maximal cardiac output (e.g., stroke volume) and blood volume may also contribute to the greater gain in VO_{2max} in the PRO group; however, we were not able to measure this. Thus, based on our findings, the greater gain in VO_{2max} in the PRO group can be partly explained by changes in lean mass and skeletal muscle oxidative capacity.

It has previously been demonstrated that endurance training induces skeletal muscle hypertrophy (43–45). We found mild but nonsignificant increases in lean mass as a result of endurance training in the CON group. In contrast, protein supplementation substantially increased lean mass after 5 wk of endurance training. Our finding that lean mass had increased after 5 wk of endurance training only when protein supplements were provided underlines the importance of adequate protein intake for skeletal muscle reconditioning during endurance training. Since endurance exercise increases muscle protein synthesis (46, 47), it is not surprising that studies have documented considerable muscle hypertrophy following prolonged endurance training (43, 45). The mechanism by which protein supplementation promotes lean mass gain is most likely the greater response following the exercise-induced stimulation in myofibrillar protein synthesis. Indeed, it has been previously shown that ingesting protein further stimulates myofibrillar muscle protein synthesis rates during recovery from a bout of cycling exercise (47). Furthermore, protein feeding following endurance exercise modulates mRNA-specific pathways involved in myogenesis and type I fiber remodeling (48). In addition to the gain in lean mass, we also observed a substantial loss of fat mass in the PRO group (-1.2 ± 0.4 kg). The loss of fat mass could have resulted from the gain in lean mass through increased resting energy expenditure, thereby eliciting a caloric deficit. Furthermore, fat mass loss was

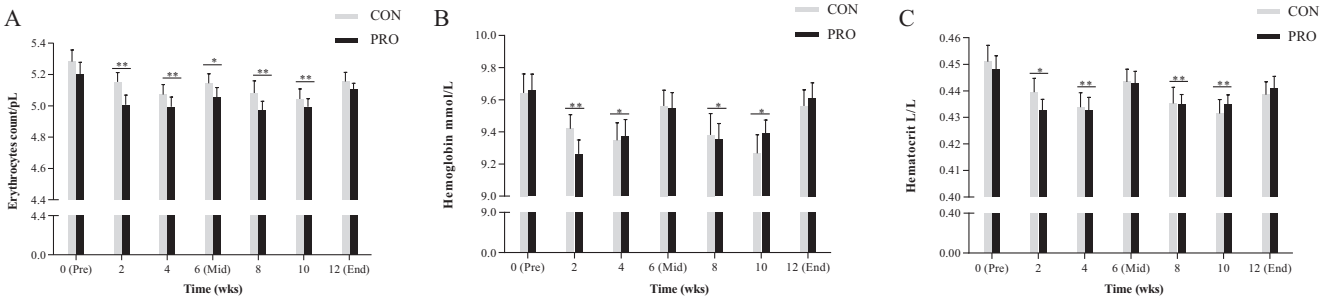

FIGURE 6 Hematological factors throughout the intervention. Erythrocytes (count/pL) (A), hemoglobin (mmol/L) (B), and hematocrit (L/L) (C) during 10-wk endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Data were analyzed using 2-way mixed ANOVA for time-by-treatment interaction for erythrocytes, $P = 0.771$, hemoglobin, $P = 0.483$, and hematocrit, $P = 0.699$. Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. $^{*}P < 0.05$ significant main effect of time compared with Pre; $^{**}P < 0.0001$ significant main effect of time compared with Pre. CON, carbohydrate supplemented; PRO, protein supplemented.

TABLE 6 Body composition[1]

	CON group ($n = 21$)			PRO group ($n = 19$)			P		
	Pre	Mid	End	Pre	Mid	End	Training	Treatment	Interaction
Lean mass, whole body, kg	61.0 ± 0.9	61.1 ± 0.9	61.4 ± 1.0	60.1 ± 1.1	61.6 ± 1.2*	61.6 ± 1.2*	<0.001	NS	0.001
Lean mass, trunk, kg	28.1 ± 0.5	28.2 ± 0.5	28.4 ± 0.5	27.7 ± 0.5	28.3 ± 0.6	28.4 ± 0.5	0.001	NS	NS
Lean mass, legs, kg	21.6 ± 0.4	21.5 ± 0.4	21.6 ± 0.4	20.9 ± 0.4	21.8 ± 0.5*	21.6 ± 0.5*	0.005	NS	0.002
Lean mass, arms, kg	7.1 ± 0.2	7.2 ± 0.2	7.1 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	NS	NS	NS
Fat mass, whole body, kg	12.8 ± 1.0	12.7 ± 1.0	12.6 ± 1.1	12.8 ± 0.7	12.2 ± 0.7*	11.6 ± 0.8*†	<0.001	NS	0.014
Fat mass, trunk, kg	7.1 ± 0.5	7.1 ± 0.6	7.1 ± 0.6	7.0 ± 0.6	6.6 ± 0.6*	6.2 ± 0.6*	0.007	NS	0.010
Fat mass, legs, kg	4.3 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.3 ± 0.2	4.2 ± 0.2	3.9 ± 0.3	<0.001	NS	NS
Fat mass, arms, kg	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	NS	NS	NS
Bone mineral density, g/cm^2	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	NS	NS	NS

[1] Values are means ± SEM. *Significantly different compared with Pre, $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; NS, $P \geq 0.05$; PRO, protein-supplemented.

inversely correlated with the gain in lean mass in the PRO group ($r^2 = 0.5$; $P \leq 0.001$). The greater gain in VO_{2max} and improved body composition as a result of protein supplementation did not increase the exercise-induced improvements in 10-km time trial performance when compared with control. Acknowledging the interindividual variation on the 10-km time trial, the present study was probably underpowered and/or of too short duration to detect potential differences in endurance performance as a result of protein supplementation. Therefore, the beneficial effects of protein supplementation on exercise performance during prolonged endurance training remain to be established and future work is needed to define the impact of the greater gain in VO_{2max} on various types of endurance performance, where duration and intensity may be of key importance.

The findings observed in the present study mirror findings of some (17, 18) but not all (16) previous studies that have determined the impact of protein supplementation on changes in VO_{2max}, skeletal muscle oxidative capacity, endurance performance, and body composition following prolonged endurance training. These discrepancies are most likely attributable to methodological differences between studies. For instance, aspects such as cohort size, applied exercise variables (e.g., type, duration, intensity), and degree of monitoring, as well as the amount, type, and timing of protein supplementation, could largely affect study findings. We speculate that in our study the vigorous intensity of the exercise training performed in combination with the type of protein supplement and the supplementation strategy may have contributed largely to the additional effects of protein supplementation. We decided to provide isocaloric supplements in both groups. Consequently, we provided the CON group with an isocaloric carbohydrate drink (28 g oligosaccharides). Hence, given that the carbohydrate beverage increased carbohydrate intake in the CON group, it cannot be stated conclusively that merely the protein was responsible for the different responses in the 2 groups. For example, omission of postexercise carbohydrate provision could have increased postexercise molecular signaling responses, thereby improving endurance training adaptations in the PRO group (49). However, it should be noted that all subjects received 2 slices of gingerbread (providing 2 × 28 g carbohydrate) immediately after every training session, implying that protein (in)availability was the key factor responsible for the findings reported here. Furthermore, analysis of habitual macronutrient intake and physical activity levels revealed no differences between the PRO and CON groups.

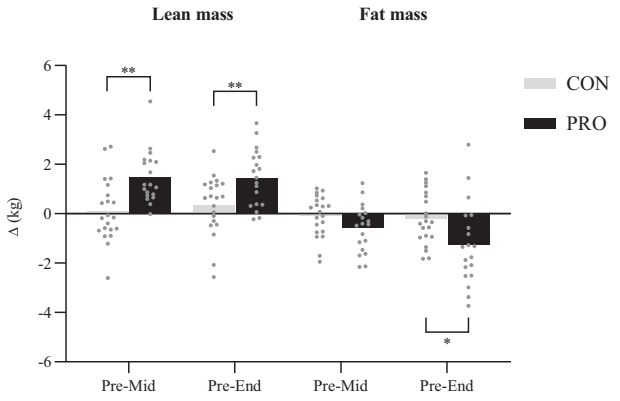

FIGURE 7 Delta changes in lean mass and fat mass from Pre-Mid and Pre-End during prolonged endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as mean group difference. Gray dots indicate individual subject responses. Data were first analyzed using 2-way mixed ANOVA to detect time-by-treatment interaction for lean mass, $P = 0.001$, and fat mass, $P = 0.015$. Unpaired t-tests were used to compare delta changes between groups from Pre-Mid and Pre-End. *$P < 0.05$ significantly different compared with CON. **$P < 0.0001$ significantly different compared with CON. CON, carbohydrate supplemented; PRO, protein supplemented.

This is to our knowledge the first double-blind randomized controlled trial with repeated measures providing a protein supplementation strategy designed to optimize protein availability to support training adaptation during prolonged endurance training. We chose to apply a supplementation strategy that theoretically should be most effective based upon previous postexercise and before-sleep protein fractional synthetic rate measurements. Provision of 30 g calcium caseinate protein after each exercise session (19, 20) and daily before sleep has previously been shown effective to increase gains in muscle mass and strength following prolonged resistance training (49). It must be noted however, that subjects in our study were provided with a protein beverage postexercise and before sleep, and based on the supplementation strategy utilized we cannot formulate inferences as to the effectiveness of the individual supplementation strategy or the potential superiority of combining postexercise and before-sleep protein supplementation. Thus, combining recent knowledge regarding the impact of protein ingestion during recovery from exercise and the impact on postexercise muscle protein synthesis rates, we show that such a supplementation

strategy effectively increases VO_{2max} and stimulates lean mass gain, thereby enhancing the efficiency of endurance training reconditioning.

In conclusion, protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion while promoting fat mass loss during 10 wk of endurance training in healthy, young males. Protein supplementation did not improve skeletal muscle oxidative capacity and endurance performance. Therefore, protein supplementation seems to form an effective dietary strategy to enhance the adaptive response to endurance training in healthy, young males.

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The authors' responsibilities were as follows—PK, LJCvL, MH and MM: designed the research; PK: conducted the research; PK: analyzed the data; PK: wrote the paper; PK: had primary responsibility for final content; and all authors: read, provided feedback, and approved the final manuscript. The authors have no conflicts of interest to declare that are directly relevant to the contents of this manuscript. LJCvL has received research grants, consulting fees, speaking honoraria, or a combination of these, from Friesland Campina and PepsiCo.

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