

Acute effects of ingestion of a novel whey-derived extract on vascular endothelial function in overweight, middle-aged men and women

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Abstract

Whey protein intake reduces CVD risk, but little is known whether whey-derived bioactive peptides regulate vascular endothelial function (VEF). We determined the impact of a whey-derived extract (NOP-47) on VEF in individuals with an increased cardiovascular risk profile. Men and women with impaired brachial artery flow-mediated dilation (FMD) (n 21, age 55 (SEM 1.3) years, BMI 27.8 (SEM 0.6) kg/m², FMD 3.7 (SEM 0.4)%) completed a randomised, cross-over study to examine whether ingestion of NOP-47 (5 g) improves postprandial VEF. Brachial artery FMD, plasma amino acids, insulin, and endothelium-derived vasodilators and vasoconstrictors were measured for 2 h after ingestion of NOP-47 or placebo. Acute NOP-47 ingestion increased FMD at 30 min (4.6 (SEM 0.5)%) and 120 min (5.1 (SEM 0.5)%) post-ingestion ($P < 0.05$, time \times trial interaction), and FMD responses at 120 min were significantly greater in the NOP-47 trial compared with placebo (4.3 (SEM 0.5)%). Plasma amino acids increased at 30 min following NOP-47 ingestion ($P < 0.05$). Serum insulin increased at 15, 30 and 60 min ($P < 0.001$) following NOP-47 ingestion. No changes were observed between the trials for plasma NO[•] and prostacyclin metabolites or endothelin-1. Ingestion of a rapidly absorbed extract derived from whey protein improved endothelium-dependent dilation in older adults by a mechanism independent of changes in circulating vasoactive compounds. Future investigation is warranted in individuals at an increased CVD risk to further elucidate potential health benefits and the underlying mechanisms of extracts derived from whey.

Key words: Vascular endothelial function: Flow-mediated dilation: Nitric oxide: Amino acids: Whey protein

CVD accounts for approximately 800 000 deaths annually and is the leading cause of morbidity and mortality in the USA⁽¹⁾. The risk of CVD is substantially increased by endothelial dysfunction⁽²⁾, a process mediated through alterations in vasomotor tone, platelet adhesion and thrombosis, all of which are regulated by vasoactive compounds⁽³⁾. Suppression of endothelial functions is an early event in the progression of atherosclerosis⁽⁴⁾ and therefore represents an important target for assessment in order to manage the risk for developing CVD.

Endothelium-dependent vasodilation can be assessed 'non-invasively' in the peripheral circulation by brachial artery flow-mediated dilation (FMD)⁽⁵⁾. Assessment of endothelial function using FMD has been suggested to represent a functional surrogate for NO[•] bioavailability in humans⁽⁶⁾,

although recent findings have demonstrated that NO[•] is not obligatory for conduit artery FMD in healthy adults⁽⁷⁾. Indeed, FMD responses are related to coronary artery endothelial function⁽⁸⁾ as well as risk factors for CVD and mortality due to CVD⁽⁹⁾. Thus, impaired FMD responses are regarded as an early manifestation in the progression of CVD⁽¹⁰⁾.

Ageing is an inevitable biological process that represents a major, independent risk factor for CVD morbidity and mortality⁽²⁾. Impaired vascular endothelial function, as demonstrated by reduced endothelium-dependent dilation, has been reported in otherwise healthy middle-aged and older adults^(11,12). Biosynthesis and/or bioavailability of NO[•] is impaired in healthy older humans and represents a critical mechanism underlying ageing-mediated endothelial

Abbreviations: %FMD, percentage of flow-mediated dilation; ACE, angiotensin-converting enzyme; ET-1, endothelin-1; FMD, flow-mediated dilation; PRE, pre-ingestion; SR_{AUC}, area under the shear rate curve; UPLC, ultra-performance liquid chromatography.

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dysfunction⁽¹³⁾. Therefore, interventions that improve NO⁻ homeostasis would not only be expected to improve endothelial function, but also the risk of CVD-related morbidity and mortality.

Bioactive peptides derived from food, especially dairy products, have been shown to exert numerous bioactivities including decreasing blood pressure^(14,15) and improving endothelial function⁽¹⁶⁾. Milk-derived peptides also inhibit angiotensin-converting enzyme (ACE) activity *in vitro*^(17,18). That chronic whey consumption in humans reduces blood pressure^(18,19) and protects against vascular dysfunction⁽¹⁹⁾ has prompted studies to examine whether its acute ingestion can regulate postprandial responses leading to impaired vascular function. Our prior work demonstrated that acute ingestion of an extract isolated from a whey protein hydrolysate (NOP-47) improved NO⁻-dependent and -independent vascular responses⁽²⁰⁾. Because our prior study was limited to young adults having a low risk for CVD⁽²⁰⁾, the present study aimed to examine whether acute ingestion of NOP-47 would improve postprandial vascular function in older adults with confirmed impaired vascular endothelial function. To this end, we conducted a randomised, placebo-controlled, cross-over study to examine the effect of acute NOP-47 ingestion on FMD responses and biomarkers of vascular function during a 2 h postprandial period.

Methods

Study design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board at the University of Connecticut. Written informed consent was obtained from all subjects. A randomised, double-blinded, placebo-controlled, cross-over study design with a washout period was conducted. Each participant reported to the laboratory for three separate visits. Participants participated in one screening visit and, if they qualified, two acute vascular testing trials in which they ingested either a whey-derived extract (NOP-47; Table 1) or placebo. To ensure standardisation between the acute vascular testing trials, participants were instructed to maintain their current level of physical activity during the study period. Additionally, participants recorded their dietary intake 1 d before the first acute trial and replicated this diet record before the second acute trial. In order to reduce confounding effects on experimental outcomes, participants were instructed to fast and avoid caffeine for 12 h, avoid alcohol, strenuous exercise, aspirin and over-the-counter medications for 24 h, avoid any vitamins and supplements for 72 h⁽²¹⁾, and to consume 1 litre of water the night before and 480 ml the morning of each trial to ensure adequate hydration.

Participants

Healthy, non-smoking men (*n* 11) and postmenopausal women (*n* 10) were recruited on the basis of age (45–65 years), BMI

Table 1. Amino acid composition of the whey extract (NOP-47)

Amino acids	g/100 g powder	5 g NOP-47*
Trp	1.33	0.07
Cystine	0.52	0.03
Met	5.05	0.25
Asp	5.46	0.27
Thr	10.74	0.54
Ser	4.29	0.21
Glu	8.21	0.41
Pro	2.30	0.12
Gly	1.18	0.06
Ala	6.86	0.34
Val	7.31	0.37
Ile	5.69	0.28
Leu	22.40	1.12
Tyr	2.31	0.12
Phe	4.41	0.22
Lys	5.79	0.29
His	1.70	0.09
Arg	1.35	0.07

* The dose used in the present study was 5 g of NOP-47 powder mixed with artificial sweetener in 300 ml water.

(25–40 kg/m²), fasting total cholesterol (<3200 mg/l) and glucose (<1200 mg/l) and resting blood pressure (<140/90 mmHg) (Table 2). In addition, participants were recreationally active and free of diabetes and other metabolic diseases, and did not use any vasoactive medications. All women were postmenopausal and those using hormone replacement therapy were excluded.

Screening visit

The screening visit consisted of anthropometric measurements, determination of fasting blood lipids and glucose (Cholestech LDX System; Cholestech Corporation), and assessment of FMD (see below). Body mass was measured to the nearest 0.1 kg on a calibrated digital scale and height (\pm 0.1 cm) was measured using a stadiometer. Waist and hip circumference was determined and recorded to the nearest 0.1 cm. Following

Table 2. Subject characteristics (*n* 21, eleven males)

(Mean values with their standard errors)

Variables	Mean	SEM
Age (years)	55.2	1.3
Height (cm)	171.6	2.7
Weight (kg)	82.4	3.2
BMI (kg/m ²)	27.8	0.6
Waist circumference (cm)	88.1	2.5
Hip circumference (cm)	104.9	1.7
SBP (mmHg)	124.0	2.5
DBP (mmHg)	80.0	1.8
HR (bpm)	61.0	1.9
TC (mg/l)	2008	55
TAG (mg/l)	1122	131
HDL (mg/l)	514	28
LDL (mg/l)	1278	54
Glucose (mg/l)	998	22

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; TC, total cholesterol.

a 15 min rest period in a supine position, blood pressure was assessed twice by auscultation and heart rate assessed by palpation.

Flow-mediated dilation

Participants were enrolled on the basis of resting FMD responses of $\leq 5.0\%$ in order to identify individuals at a higher risk of having a cardiovascular event⁽²²⁾. Brachial artery FMD was assessed by high-frequency ultrasonographic imaging. All tests were performed in a quiet, temperature-controlled room after a 20 min period in a supine position. The non-dominant arm was extended and positioned perpendicularly from the torso at the level of the heart. A rapid inflation and deflation pneumatic cuff was placed on the forearm immediately distal to the olecranon process to provide a stimulus to forearm ischaemia⁽²³⁾. Using a 5–12 MHz multi-frequency linear array transducer attached to a high-resolution ultrasound machine (T3000; Terason), the brachial artery was imaged longitudinally in the distal third of the upper arm with clear visualisation of the anterior and posterior vessel walls. Simultaneous assessment of continuous Doppler velocity using the ultrasound machine was performed at an insonation angle of 60°. After optimisation of the image and blood velocity signal, the probe was held stable and end-diastolic baseline brachial artery images were recorded for 1 min. The forearm cuff was then inflated to 200 mmHg for 5 min using a rapid cuff inflator (Hokanson E20; D.E. Hokanson, Inc.) to occlude the brachial artery, and then released. Diameter and blood velocity recordings resumed 1 min before cuff deflation and continued for 3 min thereafter. Ultrasound images were recorded at a rate of five frames/s using screen capture software (Camtasia Studio; TechSmith Corporation) and converted into an AVI (audio video interleave) file. Anatomical measurements were made and internal landmarks identified to ensure placement of the transducer in the same location on the arm during subsequent measurements/visits. Off-line analyses of diameters and velocities were performed using automated edge-detection software with end-diastolic gating (Medical Imaging Applications). All vascular measurements and analyses were performed by the same technician.

Using a three-frame smoothing average, post-occlusion changes in diameter were determined. The peak post-occlusion diameter was determined as the highest three-frame average. Relative (%) and absolute (mm) brachial artery FMD was calculated from the preceding baseline diameter. From end-diastolic synchronised diameter (D ; mm) and velocity data (V ; m/s), shear rate (an estimate of shear stress without viscosity; per s) was calculated as four times mean blood velocity per vessel diameter⁽²⁴⁾. The shear rate stimulus responsible for endothelium-dependent FMD following cuff deflation was calculated in accordance with recent findings⁽²⁵⁾. The area under the shear rate curve (SR_{AUC} ; arbitrary units), calculated for data up to the point of maximal post-occlusion diameter⁽²⁵⁾, was determined to quantify the 'relevant' hyperaemic stimulus responsible for the peak FMD response. The relative peak FMD response was normalised to shear rate and expressed as the peak FMD: SR_{AUC} ratio (arbitrary units).

Acute vascular testing protocol

The two acute trials occurred at the same time of the day separated by at least a 1-week washout period. Following assessment of resting heart rate and blood pressure, fasting measurement of FMD was determined. Next, a flexible catheter was inserted into an antecubital vein and after a 15 min supine stabilisation period, blood samples were collected from a three-way stopcock connected to the end of the catheter for fasting baseline and subsequent postprandial biochemistry measurements. Immediately following these baseline measurements, subjects consumed a single pre-packaged dose (5 g) of either NOP-47 with artificial sweetener or placebo mixed in water. NOP-47 is a proprietary extract isolated from a whey protein hydrolysate (Glanbia Nutritionals). The placebo was identical except the packets contained only artificial sweetener (aspartame and acesulfame potassium). Subjects ingested the beverage within 3 min, after which a timer was started for the 2 h postprandial testing protocol. A questionnaire to address subjective symptoms and side effects associated with each supplement was administered at the end of the study.

Postprandial brachial artery FMD measurements were made at 30, 60 and 120 min, while blood samples were obtained at 15, 30, 60, 90 and 120 min post-ingestion. Blood pressure was assessed by the same investigator at 15, 45, 75, 105 and 125 min post-ingestion. Subjects remained supine in a comfortable position for the entire duration of the test.

Blood collection and biochemical analyses. Whole blood was collected into tubes with no additives (serum), or tubes containing EDTA, or lithium heparin and centrifuged (1500 g, 15 min, 4°C). Before centrifugation, haematocrit was determined from the whole blood in duplicate using a microcapillary reader. Serum and plasma were transferred into storage tubes, snap frozen in liquid N₂ and stored at -80°C for future analysis. Samples for each assay were analysed in duplicate.

The liquid glucose hexokinase assay was used to measure plasma glucose in accordance with the manufacturer's instructions (Pointe Scientific, Inc.). Glucose concentrations were determined at 340 nm on a spectrophotometer (CV 4.5%, VersaMax Microplate Reader; Molecular Devices Corporation). Insulin was measured from serum using a commercially available ELISA kit (CV 3.9%, CalBiotech, Inc.) in accordance with the manufacturer's instructions.

Total nitrite/nitrate (NO_x), an index of NO[•] production, was determined by the Griess reaction using a colorimetric method described by Giustarini *et al.*⁽²⁶⁾ with minor modification (CV 3.0%). Briefly, plasma samples were filtered using 10 kDa molecular-weight cut-off filters before the analysis in order to reduce background absorbance. Diluted plasma filtrate (1:3) was mixed on a ninety-six-well plate with nitrate reductase (Sigma-Aldrich) and a solution containing NADPH and FAD, and then incubated for 90 min at room temperature. Following this, 200 μl of Griess reagent were added to the wells and the plate was incubated for 30 min in the dark. Following incubation, samples were read at 540 nm on a microplate reader (SpectraMax M2; Molecular Devices

Corporation). The major metabolite of the vasodilatory molecule prostacyclin, 6-keto PGF_{1α} (CV 2.9%; Cayman Chemical Company), and the potent vasoconstrictor endothelin-1 (ET-1, CV 4.1%; R&D Systems, Inc.) were measured in the plasma using commercially available ELISA kits. Serum ACE activity was determined before and at 30, 60 and 120 min post-ingestion using an enzymatic assay, with minor modification (CV 1.3%; Alpco Immunoassays). Briefly, 250 μl of the synthetic substrate FAPGG were added to each well containing 25 μl calibrator, control or unknown serum, and incubated at 37°C for 5 min. The wavelength was then read twice at an interval of exactly 10 min at 340 nm to measure the decrease in the absorbance of the cleavage reaction mediated by ACE.

Plasma amino acids were analysed from blood samples obtained at baseline and at 30, 60 and 120 min postprandial. Untreated Na heparin plasma samples were stored at -80°C and processed using the protocol outlined below upon thawing at room temperature. The samples were initially centrifuged at 10 000 g for 5 min to remove any particulate matter from the fluid. Next, 25 μl of the supernatant were diluted 4-fold with a 1.25 μM internal standard solution of 3,5-diiodo-tyrosine. The resultant mixture was then transferred to a 3 kDa molecular-weight cut-off filter (Pall Corporation) and centrifuged for 10 min at 7500 g. The low-molecular-weight filtrate was then diluted 12.5-fold in the appropriate mobile phase and used for amino acid analysis.

Amino acids were quantified by ultra-performance liquid chromatography tandem MS (UPLC-MS/MS) using an Acquity Ultra-Performance LC system (Waters) coupled to a Waters Micromass® Quattro Premier triple quadrupole instrument (Waters). The UPLC-MS/MS experimental parameters for determining underivatized amino acids have been detailed elsewhere^(27–29). Data acquisition was performed using MassLynx™ 4.1 and QuanLynx™ software (Waters).

Statistical analyses

Statistical analyses were performed using STATISTICA (StatSoft, Inc.). All data were initially evaluated for homogeneity of variance and normality. Dependent variables that were not normally distributed were log transformed using the natural base to achieve normality. Untransformed values are reported with their significance levels because there were no qualitative differences in the results and they permit more meaningful interpretations than transformed data. Differences between the acute trials for dietary intakes and pre-ingestion values were evaluated using two-tailed Student's paired *t* tests. Treatment and time effects for postprandial vascular and haematological responses were evaluated using two-way repeated-measures ANOVA with time points as the within effect and trial as the between effect. When main or interaction effects were present, Fisher's least significant difference *post hoc* test was used to assess pairwise comparisons. Multiple linear regression, controlling for within-subject repeated measures, was used to calculate correlation coefficients as described by Bland & Altman⁽³⁰⁾. The CV (within-subject SD/within-subject mean × 100) for the baseline diameter, peak diameter and percentage of FMD (%FMD)

were 1.2, 1.6 and 32.2%, respectively, and are comparable with previous investigations^(31–33). The α -level for significance was set at ≤ 0.05 .

Results

Screening visit

A total of twenty-one middle-aged adults (Table 2) qualified to participate in the two supplement trials. None of the variables assessed during the screening visit differed from either of the two supplement trials at baseline ($P > 0.05$).

Acute ingestion trials

No adverse events were reported by subjects during either trial. Additionally, no differences in dietary macronutrient composition were found between the trials (data not shown), indicating that participants maintained similar dietary patterns before each trial.

Vascular function. Pre-ingestion (PRE) FMD was similar between the trials ($P = 0.144$; Fig. 1(a)). A significant time and interaction effect for FMD ($P < 0.05$) indicated that FMD responses decreased from PRE at 60 min during the placebo trial. In addition, FMD responses during the NOP-47 trial increased at 30 min ($P < 0.05$) and 120 min ($P < 0.001$) relative to PRE. Moreover, FMD at 120 min was higher following NOP-47 ingestion than the corresponding placebo time point ($P < 0.05$).

Pre-occlusion brachial artery diameters were not significantly different before ingestion of NOP-47 and placebo (Table 3; Fig. 1(b)). However, there was a significant main effect of time ($P = 0.002$), and *post hoc* analyses indicated that pre-occlusion brachial artery diameters decreased slightly from pre-ingestion at 30 and 120 min post-ingestion. No differences were detected between the trials ($P = 0.52$). In order to address the differences in pre-occlusion diameter within the trials, FMD was calculated as an absolute change. Similar to the above, a significant time ($P = 0.020$) and interaction effect ($P = 0.054$) for the absolute change in FMD indicated that FMD responses decreased ($P = 0.031$) from PRE (0.18 (SEM 0.02) mm) at 60 min (0.14 (SEM 0.02) mm) during the placebo trial. Compared with PRE (0.16 (SEM 0.02) mm), absolute FMD responses during the NOP-47 trial increased at 30 min (0.19 (SEM 0.02) mm; $P = 0.052$) and 120 min (0.21 (SEM 0.02) mm; $P < 0.01$). Moreover, the absolute change in FMD at 120 min was higher ($P = 0.038$) following NOP-47 ingestion than the corresponding placebo time point (0.18 (SEM 0.02) mm and 0.21 (SEM 0.02) mm for 120 min placebo time point *v.* 120 min NOP-47 time point, respectively). No differences were detected between the trials for the absolute change in FMD ($P = 0.36$). There were no differences in peak brachial artery diameter over time or between the trials ($P > 0.05$; Table 3; Fig. 1(b)).

There was a main effect of time for pre-occlusion shear rate in that PRE had a significantly higher shear rate compared with 30 and 60 min post-ingestion for both trials ($P < 0.05$; Table 3). The AUC of the post-occlusion shear rate calculated until the



time-to-peak diameter measurement was significantly higher at PRE compared with the 30 and 60 min time points in both trials ($P < 0.05$; Fig. 2). In addition, a significant main effect for trial ($P < 0.05$) was determined. However, *post hoc* analyses revealed no significant differences between the trials when data were collapsed ($P = 0.31$). A significant main effect of time for mean arterial pressure was detected ($P < 0.05$). Compared with PRE values (93.9 (SEM 2.0) mmHg), mean arterial pressure increased ($P < 0.05$) slightly at 125 min post-ingestion (95.3 (SEM 1.9) mmHg).

There was a main effect of time for FMD normalised to the magnitude of the shear rate stimulus (%FMD/SR_{AUC}; $P < 0.05$). *Post hoc* analyses indicated a trend for this ratio to increase at 30 min ($P = 0.069$) and 120 min ($P = 0.065$) post-ingestion, with no differences detected between the trials. There was no

difference in the time-to-peak diameter over time or between the trials.

Haematological responses. Pre-ingestion biomarker concentrations did not significantly differ between the trials (Table 3). Compared with PRE, haematocrit increased slightly at 90 min post-ingestion but not at any other postprandial time point ($P < 0.01$). Plasma glucose was unaffected by acute ingestion. Serum insulin increased significantly at 15 min ($P < 0.01$), 30 min ($P < 0.001$) and 60 min ($P < 0.001$) following NOP-47 ingestion (Fig. 3). The 15, 30 and 60 min insulin values were significantly higher following NOP-47 ingestion than the corresponding placebo time points ($P < 0.01$ for time \times trial interaction).

A significant main effect for trial ($P < 0.05$) and a trend for a time effect ($P = 0.07$) was determined for plasma NO_x. When

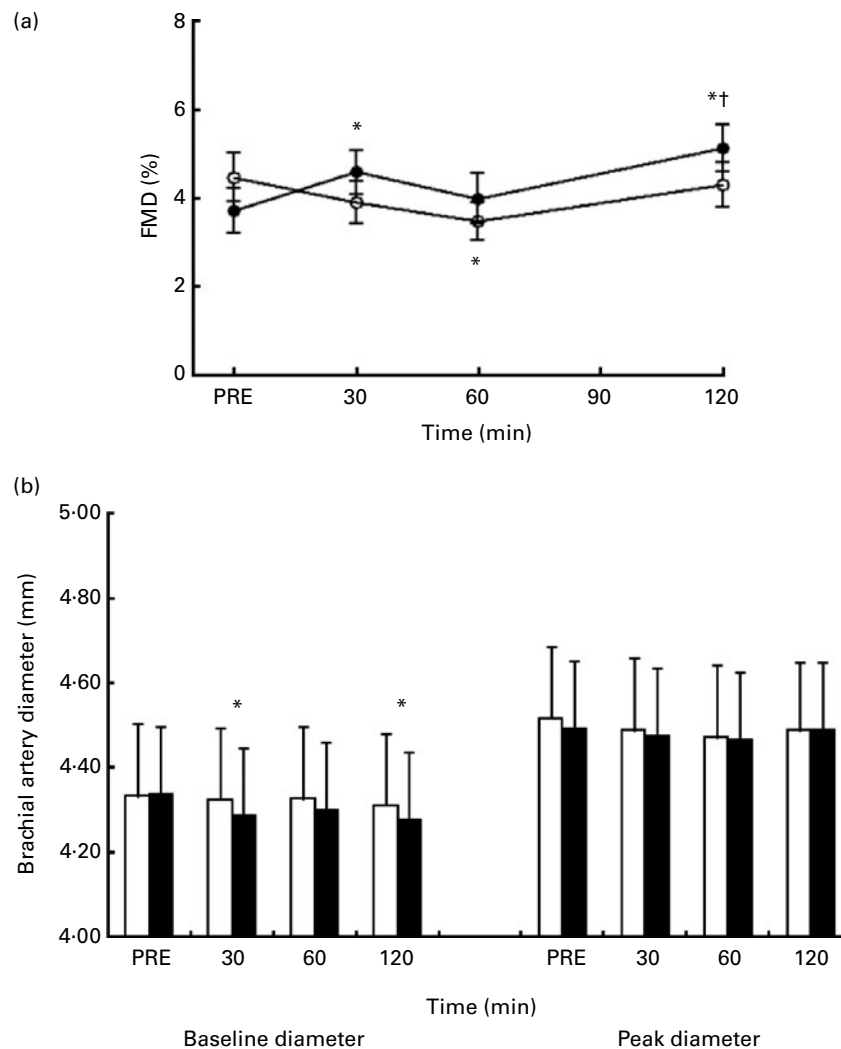


Fig. 1. Responses (a) for peak flow-mediated dilation (FMD) pre-ingestion (PRE) and following ingestion of placebo (○) or whey-derived extract (NOP-47; ●). Values are means, with standard errors represented by vertical bars. * Mean value was significantly different from that of PRE ($P < 0.05$; Fisher's least significant difference (LSD) *post hoc* test). † Mean value was significantly different from that of the 120 min placebo time point ($P < 0.05$; Fisher's LSD *post hoc* test). There were significant time ($P < 0.05$) and trial \times time interaction ($P < 0.05$) effects. No effect was detected for trial ($P = 0.43$). (b) Baseline and peak brachial artery diameters PRE and following ingestion of placebo (□) or NOP-47 (■). Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of PRE ($P < 0.05$; Fisher's LSD *post hoc* test). There was a significant main effect of time ($P < 0.01$) for baseline diameter, but no effect for trial ($P = 0.52$). There was no significant trial \times time interaction effect ($P = 0.29$). For peak diameter there was no significant effect for time ($P = 0.18$), or trial ($P = 0.78$) and no significant trial \times time interaction effect ($P = 0.89$).

concentrations were calculated as the change from PRE values, no main effects were detected (Fig. 4(a)).

Significant main effects for time ($P < 0.05$) and trial ($P = 0.05$) were detected for the major metabolite of prostacyclin, 6-keto $\text{PGF}_{1\alpha}$. Compared with PRE, 6-keto $\text{PGF}_{1\alpha}$ increased over time in both trials ($P < 0.05$) with higher values at 15 and 120 min post-ingestion ($P < 0.05$). When concentrations were calculated as the change from PRE values, no main effects were detected (Fig. 4(b)). Plasma levels of the potent vasoconstrictor ET-1 increased transiently at 15 and 30 min post-ingestion ($P < 0.05$) with no difference detected between the trials (Fig. 5). Serum ACE activity was unaffected in either trial ($P > 0.05$).

Pre-ingestion plasma amino acid concentrations were not different between NOP-47 and placebo. All concentrations of plasma amino acids, except for citrulline, increased after 30 min following ingestion of NOP-47 and returned to fasting levels within 60 or 120 min (Table 4). Plasma citrulline significantly decreased from PRE values at 30 min ($P < 0.0001$), 60 min ($P < 0.0001$) and 120 min ($P < 0.0001$) post-ingestion for both trials. In general, the magnitude of the increase in plasma amino acids paralleled the amino acid composition of NOP-47 as exemplified by the 2-fold increase in plasma leucine at 30 min. Concentrations of serine, threonine, asparagine, glutamic acid, valine, methionine, leucine, isoleucine, phenylalanine, tryptophan, glutamine, arginine, glycine,

Table 3. Vascular and haematological responses to acute ingestion of whey-derived extract (NOP-47) and placebo

(Mean values with their standard errors)

Variables	NOP-47		Placebo	
	Mean	SEM	Mean	SEM
Pre-occlusion shear rate (per s)				
Pre-ingestion	171.7	13.4	158.4	10.6
30 min*	152.8	13.7	129.8	9.1
60 min*	146.3	14.8	133.9	9.8
120 min	158.7	12.3	141.4	11.2
Pre-occlusion brachial artery diameter (mm)				
Pre-ingestion	4.34	0.16	4.33	0.17
30 min*	4.29	0.16	4.33	0.17
60 min	4.30	0.16	4.33	0.17
120 min*	4.28	0.16	4.31	0.17
Peak brachial artery diameter (mm)				
Pre-ingestion	4.49	0.16	4.52	0.17
30 min	4.47	0.16	4.49	0.17
60 min	4.46	0.16	4.47	0.17
120 min	4.49	0.16	4.49	0.16
Shear rate AUC (arbitrary units)				
Pre-ingestion	26473.2	2598.7	23773.0	2216.2
30 min*	22025.9	2285.9	19308.2	1892.9
60 min*	22329.7	1929.8	17543.7	1467.8
120 min	24422.4	2198.4	22043.3	1589.5
TTP (s)				
Pre-ingestion	48.0	3.6	49.2	3.3
30 min	43.2	3.4	46.5	3.9
60 min	49.0	4.3	41.6	3.4
120 min	49.4	4.6	47.1	4.6
Haematocrit (%)				
Pre-ingestion	41.0	0.5	40.2	0.7
15 min	41.1	0.5	40.6	0.6
30 min	41.3	0.5	40.4	0.6
60 min	41.0	0.6	40.4	0.7
90 min*	41.4	0.6	40.9	0.6
120 min	41.1	0.6	40.6	0.6
Plasma glucose (mg/l)				
Pre-ingestion	896	21	904	25
15 min	898	21	911	27
30 min	907	22	896	26
60 min	885	24	902	26
90 min	891	19	910	23
120 min	895	22	912	22
Serum ACE activity (U/l)†				
Pre-ingestion	37.5	3.3	39.0	3.8
30 min	37.4	3.4	37.9	3.7
60 min	36.3	3.8	35.3	3.4
120 min	35.0	2.6	35.8	3.0

AUC, area under the curve; TTP, time-to-peak diameter; ACE, angiotensin-converting enzyme.

*There was a significant main time effect ($P < 0.05$) from pre-ingestion.

†1 U = 0.017 μkat .

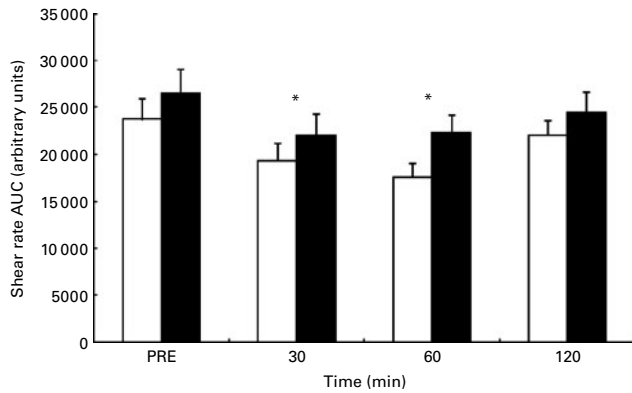


Fig. 2. Shear rate area under the curve (AUC) prior to pre-ingestion (PRE) and following ingestion of placebo (□) or whey-derived extract (NOP-47; ■). Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of PRE ($P < 0.05$; Fisher's least significant difference *post hoc* test). There were significant effects for time ($P < 0.01$) and trial ($P = 0.048$) but no significant trial \times time interaction effect ($P = 0.784$).

aspartic acid, citrulline, alanine, proline, ornithine and cysteine showed significant interaction effects ($P < 0.05$, time \times trial), indicating that ingestion of NOP-47, but not placebo, resulted in a rapid increase in plasma amino acid levels.

Multiple linear regression, controlling for within-subject repeated measures, did not indicate a relationship between FMD and any haematological measure.

Discussion

The findings of the present study indicate that acute ingestion of an extract derived from whey protein was rapidly absorbed and improved endothelium-dependent dilation in older adults with vascular endothelial dysfunction. Impaired vascular endothelial function has been reported in healthy, sedentary middle-aged and older adults^(11,12). Therefore, we sought to determine the acute vascular and haematological effects of ingestion of a novel extract derived from whey in a population at risk for CVD. Similar to previous data from our laboratory in young healthy men and women⁽²⁰⁾, acute ingestion of NOP-47 significantly increased postprandial FMD measured at 30 and 120 min post-ingestion in this group of participants at increased risk for CVD. The clinical significance of the transient changes in FMD in the present study of the order of 1–1.5% is uncertain. In asymptomatic low-risk individuals, lower FMD levels have been shown to predict future adverse cardiovascular events (a hazard ratio of 1.12 for every 1% decrease in FMD)⁽²²⁾. Taking these findings into account, the transient changes in FMD seen in the present study would be viewed as significantly altering cardiovascular risk status and clinically relevant. The increase in FMD after NOP-47 ingestion was independent of changes in circulating metabolites of NO[•], prostacyclin and ET-1, as these biomarkers were not different between the NOP-47 and placebo trials. However, local changes produced by the whey extract in vascular endothelium were not evaluated; therefore, the mediatory role of NO[•], prostacyclin and ET-1 in the observed transient increase of FMD cannot be excluded. As ageing is

associated with enhanced oxidative stress and inflammation^(34,35), it cannot be ruled out that NOP-47 ingestion transiently augmented FMD by attenuating elevated levels of oxidative stress and/or inflammation. However, previous research from our laboratory⁽²⁰⁾ demonstrated that ingestion of NOP-47 enhanced FMD in the context of minimal changes in glucose and markers of oxidative stress, inflammation and antioxidant status in healthy young adults, making it unlikely that changes in oxidative stress contributed to the increase in FMD following NOP-47 ingestion in the present investigation.

In the placebo trial, we found a small but significant decrease in peak FMD at 60 min post-ingestion compared with baseline (mean absolute reduction 1.0%), an effect not seen at the same time point following NOP-47 ingestion. The shorter time allowed between the 30 and 60 min FMD tests may explain this transient impairment. The present findings are in contrast to previous reports demonstrating no impairment in FMD when multiple FMD tests were performed closely together^(33,36). As reductions in the bioavailability of NO[•] and increases in oxidative stress and inflammation play important roles in age-related endothelial dysfunction^(34,35), perhaps the older age of the participants studied in the present investigation accounts for our findings of slightly decreased FMD at 60 min following placebo ingestion. These findings might suggest that aged arteries are more vulnerable to ischaemia/reperfusion due to increased levels of oxidative stress and inflammation. Utilising a longer period of forearm ischaemia (20 min), Devan *et al.*⁽³⁷⁾ demonstrated that sedentary ageing is associated with a greater magnitude of FMD impairment and delayed recovery from ischaemia/reperfusion compared with younger and age-matched physically active individuals. We are not aware of any investigations that have examined the impact of repetitive reactive hyperaemia on FMD assessed

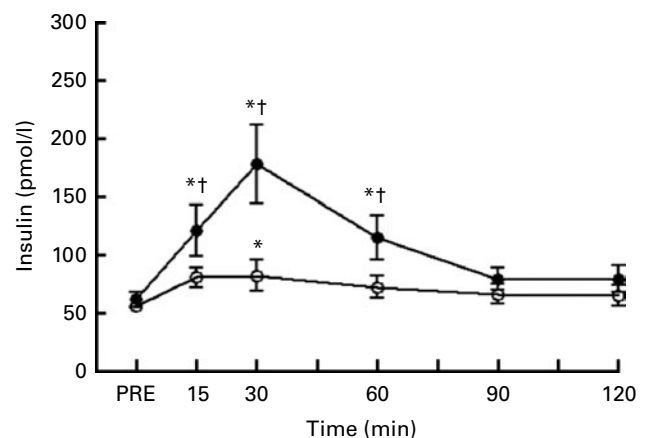


Fig. 3. Postprandial serum insulin responses to ingestion of a whey-derived extract (NOP-47; ●) or placebo (○). Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that for pre-ingestion (PRE) ($P < 0.05$; Fisher's least significant difference (LSD) *post hoc* test). † Mean value was significantly different from that for placebo at the same time point ($P < 0.01$; Fisher's LSD *post hoc* test). There were significant trial ($P < 0.01$), time ($P < 0.001$) and trial \times time interaction ($P < 0.001$) effects.

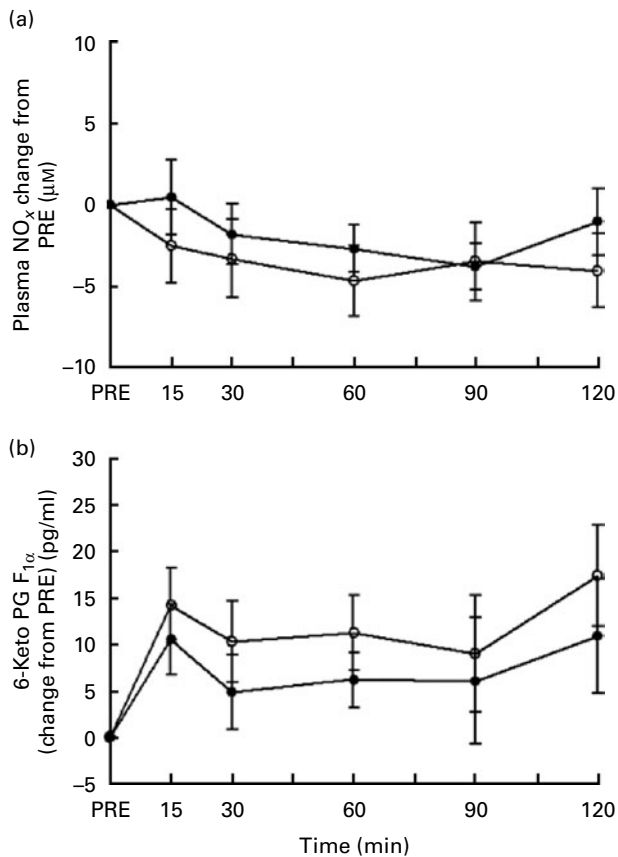


Fig. 4. (a) Plasma total nitrites/nitrates (NO_x) and (b) 6-keto PGF_{1α}, normalised to pre-ingestion (PRE), responses to ingestion of a whey-derived extract (NOP-47; ●) or placebo (○). Values are means, with their standard errors represented by vertical bars. For plasma NO_x, there was no significant effect for trial ($P=0.49$) or time ($P=0.24$) and no significant trial \times time interaction effect ($P=0.43$) when concentrations were calculated as the change from PRE values. For 6-keto PGF_{1α}, there was no significant effect for trial ($P=0.10$) or time ($P=0.27$) and no significant trial \times time interaction effect ($P=0.77$) when concentrations were calculated as the change from PRE values.

following 5 min of ischaemia in older adults with an increased cardiovascular risk profile.

The results of the present study are in agreement with those of Nerla *et al.*⁽³⁸⁾ and support the finding that two consecutive reactive hyperaemia-induced increases in shear stress performed in close succession transiently impairs FMD. In the study by Nerla *et al.*, the reduction in FMD was associated with increased levels of the endogenous NO synthase inhibitor asymmetric dimethylarginine. Interestingly, when L-arginine was infused intravenously for 20 min before the two consecutive FMD measures, the impairment in the second FMD was prevented. These results suggest that NO[•] depletion induced by increased levels of NO synthase inhibitors (i.e. asymmetric dimethylarginine) and/or reduced substrate availability impairs FMD when subsequent measurements are performed within close proximity. In the present study, NOP-47 ingestion prevented the decline in FMD at 60 min, despite the extract being low in arginine (<2%). Plasma concentrations of arginine increased transiently following the ingestion of NOP-47, but not placebo, demonstrating that

increased substrate availability may be responsible for the preserved FMD seen at 60 min in the NOP-47 trial.

Compared with pre-ingestion, a significant decrease in the post-occlusion shear rate stimulus magnitude (i.e. SR_{AUC}) was detected at 30 and 60 min post-ingestion, with no differences between the trials. Similar results were obtained in young individuals who were subjected to four consecutive FMD tests separated by a mean inter-trial time of 13.5 min⁽³³⁾. Despite this decreased stimulus, acute NOP-47, but not placebo, ingestion significantly increased FMD at 30 min. The present data of a smaller shear rate stimulus in conjunction with an augmented FMD response would be interpreted as an increase in endothelial sensitivity to shear stress and improved endothelial function. Additionally, normalisation of the FMD response to the shear stress stimulus (%FMD/SR_{AUC}) did not clarify our interpretation of increased FMD following NOP-47 ingestion. According to recent guidelines⁽³⁹⁾, there must be at least a moderate correlation between FMD and the shear rate stimulus to justify normalisation of the response. Multiple linear regression, controlling for within-subject repeated measures, indicated a weak correlation between %FMD and shear rate AUC (combined trials: mean slope 0.0004, $r=0.191$, $P=0.020$), a finding that has previously been reported in older adults⁽⁴⁰⁾.

Plasma insulin is elevated following L-arginine administration and may account for reported improvements in FMD following L-arginine supplementation^(41,42). Importantly, in the study by Gates *et al.*⁽⁴³⁾, the post-infusion FMD measurement (40 min post-infusion) was timed to isolate the effect of L-arginine from that of insulin, therefore reflecting an effect of L-arginine that is insulin-independent. In the present study, we measured FMD at 30, 60 and 120 min post-ingestion with insulin returning to baseline levels within 90 min. Therefore, the significantly greater FMD value detected at 120 min following NOP-47 ingestion suggests an insulin-independent mechanism.

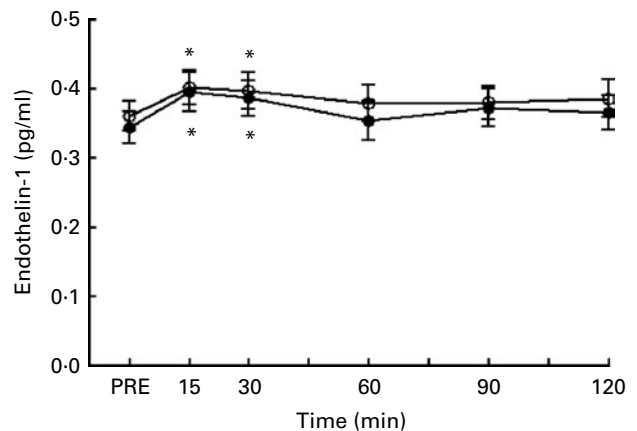


Fig. 5. Plasma endothelin-1 responses to ingestion of a whey extract (NOP-47; ●) or placebo (○). Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that for pre-ingestion (PRE) ($P<0.05$). There was a significant effect for time ($P<0.05$) but no significant trial ($P=0.26$) or trial \times time interaction ($P<0.88$) effect.

Table 4. Fold increase in plasma amino acid concentrations that were significant due to the ingestion of a whey-derived extract (NOP-47)

Amino acids	Pre-ingestion	30 min	60 min	120 min	Time	Interaction
Ser	1.00	1.21	1.14	1.02	0.000	0.000
Thr	1.00	1.34	1.30	1.13	0.000	0.000
Asn	1.00	1.20	1.14	0.03	0.000	0.000
Glu	1.00	1.23	1.18	1.03	0.100	0.007
Val	1.00	1.33	1.27	1.06	0.000	0.000
Met	1.00	1.91	1.67	1.27	0.000	0.000
Leu	1.00	2.07	1.85	1.33	0.000	0.000
Ile	1.00	1.72	1.49	1.03	0.000	0.000
Phe	1.00	1.30	1.19	1.03	0.000	0.000
Trp	1.00	1.16	1.12	0.95	0.000	0.000
Gln	1.00	1.12	1.15	1.07	0.000	0.009
Arg	1.00	1.16	1.13	1.05	0.000	0.000
Gly	1.00	1.04	1.01	0.99	0.023	0.003
Asp	1.00	1.42	1.13	1.02	0.004	0.010
Cit	1.00	0.92	0.80	0.92	0.000	0.000
Ala	1.00	1.14	1.14	1.03	0.000	0.000
Pro	1.00	1.10	1.08	0.99	0.000	0.000
Orn	1.00	1.08	1.06	1.03	0.004	0.000
Cys	1.00	1.03	1.02	0.97	0.000	0.040

To define the NOP-47-mediated activity on vasodilation, we measured NO[•] status by evaluating total nitrite and nitrate levels (NO_x), the stable metabolites of NO[•]. When concentrations were calculated as the change from pre-ingestion values, no main effects were detected, indicating the absence of an effect of NOP-47 ingestion on circulating metabolites of NO[•] in these middle-aged individuals (Fig. 4(a)). Although NO_x correlates with FMD, the assay is not specific for endothelial NO[•] production and could also reflect NO[•] derived from neuronal and inducible NO[•] synthase⁽⁴⁴⁾. Alternatively, alterations in the balance between vasodilators (e.g. bradykinin, adenosine, vascular endothelial growth factor and prostacyclin) and vasoconstrictors (e.g. endothelin, prostanoids and angiotensin II) have been suggested to contribute to the FMD response⁽⁴⁵⁾.

In order for oral ingestion of whey peptides to exert hypotensive or other biological effects *in vivo*, it must be absorbed intact and be transported to the target tissue while escaping destruction from intestinal brush-border or serum peptidases. Indeed, a specific whey-derived tripeptide with known high, *in vitro* ACE-inhibitory activity⁽⁴⁶⁾ has been demonstrated to be absorbed intact following oral ingestion with a plasma elimination half-life of approximately 30 min⁽⁴⁷⁾. The present data indicate that following acute ingestion of the whey-derived extract, concentrations of several amino acids, including the branched-chain amino acids, showed significant time and interaction effects, with increases detected at 30 min and concentrations returning to baseline levels at 60 or 120 min. In general, the magnitude of the increase in plasma amino acids paralleled the amino acid composition of NOP-47 (Table 1) as exemplified by the 2-fold increase in plasma leucine at 30 min. These individual amino acid data indicate that NOP-47 is quickly digested resulting in a rapid release of amino acids into the circulation, which reflects the amino acid composition of the extract. However, it cannot be determined from these data if specific bioactive peptides are absorbed intact that may potentially explain our *in vivo*

biological effects. It is currently unknown as to which components of whey proteins (i.e. individual whey protein, peptide fractions, amino acids or synergistic actions among them) mediate the physiological effects of whey.

A possible mechanism by which whey proteins might improve endothelial function is through ACE inhibition. Human clinical trials have shown improvement in endothelial function in patients taking ACE inhibitors (reviewed in Tousoulis *et al.*⁽⁴⁸⁾), which could be the result of pleiotropic effects of ACE inhibitors on the vascular endothelium⁽⁴⁹⁾. Both decreased production of the vasoconstrictor angiotensin II and inhibited degradation of the vasodilatory molecule bradykinin occur with ACE inhibition, shifting the endothelial phenotype towards vasodilation. Dairy milk proteins inhibit ACE activity, with dairy whey proteins, in particular, exerting an anti-hypertensive effect *in vitro*^(17,18). However, in the present study, we were unable to detect an impact of NOP-47 ingestion on ACE activity measured in serum obtained from healthy human subjects. These data suggest that the transient increase in FMD demonstrated in the present study was not mediated via the inhibition of ACE.

Compared with pre-ingestion, the major metabolite of prostacyclin (PGI₂), 6-keto PGF_{1α}, increased over time in both trials with higher values detected at 15 and 120 min post-ingestion. When concentrations were calculated as the change from pre-ingestion values, no main effects were detected. The rate of PGI₂, which is the principal vascular prostanoid produced in the human vasculature⁽⁵⁰⁾, secretion into the circulation coupled with its short half-life indicate that PGI₂ functions locally⁽⁵¹⁾. Venepuncture may cause the release of PGI₂ which will artifactually increase the 6-keto PGF_{1α} concentration in the plasma⁽⁵²⁾.

Plasma levels of the potent vasoconstrictor ET-1 increased transiently at 15 and 30 min in both trials. Insulin stimulates secretion of the vasoconstrictive polypeptide ET-1 via the activation of mitogen-activated protein kinase-dependent branch of insulin signalling pathways⁽⁵³⁾. A previous study

demonstrated that insulin at physiological concentrations did not alter plasma ET-1 in healthy individuals, but did stimulate its secretion from endothelial cells *in vitro*⁽⁵⁴⁾. Since ET-1 mainly exerts paracrine and autocrine effects, serum concentrations may not completely reflect its bioactivity. The findings of the present study using multiple sampling during the postprandial period indicate that a transient increase in plasma concentrations of ET-1 does occur that is not related to increased insulin levels as plasma insulin increased to a greater extent following NOP-47 ingestion. Minor alterations in plasma volume in the present study induced by fluid ingestion and/or phlebotomy may possibly explain the small, transient increases detected in plasma ET-1 concentration, an effect demonstrated previously⁽⁵⁵⁾.

There are several limitations to the present study that should be mentioned. First, subjects were screened for impaired FMD based upon values obtained from a previous investigation⁽²²⁾. In the present study, participants were enrolled on the basis of resting FMD responses of $\leq 5.0\%$. This cut-off is consistent with the work of Shimbo *et al.*⁽²²⁾ indicating that low FMD responses ($< 7.5\%$) predict cardiovascular events in asymptomatic, lower-risk individuals at 36 months follow-up. In contrast to the present study where FMD was elicited following temporary occlusion of the forearm, Shimbo *et al.* used a FMD protocol that involved occluding the upper arm. Studies have indicated that forearm occlusion yields lower FMD values of the order of 2.5%^(56,57). Thus, we used a cut-off of 5%. The inclusion of a younger, control group with preserved FMD responses, along with a comparison of additional endothelial function measures at baseline, would have further confirmed endothelial dysfunction in the subjects investigated. Endothelium-independent dilation was not assessed so we cannot rule out an effect of the extract on vascular smooth muscle cells, as shown *in vitro*⁽⁵⁸⁾. Furthermore, even though no differences in dietary macronutrient composition were found between the trials, dietary intake of nitrate or nitrite was not controlled for which may have added variability to the present NO_x results⁽⁵⁹⁾. Despite this, we did not observe differences in pre-ingestion NO_x concentrations between the trials in the present study. Finally, the use of an iso-nitrogenous placebo, such as intact whey protein, would have helped us to further determine whether the effects seen in the two studies were due to a bioactive peptide specific to NOP-47 or simply by stimulation of insulin secretion by ingestion of whey⁽⁶⁰⁾. In support of an insulin-independent mechanism, insulin was not related to %FMD in either trial as indicated by multiple regression ($P=0.768$ and $P=0.884$ for placebo and NOP-47, respectively). These data suggest that differences in insulin responses between the two trials were not related to our observed changes in FMD.

The results of the present randomised, cross-over study suggest that in older individuals with impaired endothelial function (i.e. low FMD), acute ingestion of an extract derived from whey protein transiently improves endothelium-dependent vasodilation. The increase in FMD after whey extract ingestion was independent of changes in insulin and circulating metabolites of NO_x, prostacyclin and ET-1. The extract investigated in this small sample of healthy,

older individuals could be of clinical value as a potential therapy for patients with vascular pathologies who are at an increased CVD risk. Furthermore, studies investigating the potential of whey-derived extracts to attenuate the transient vascular impairment commonly demonstrated during the postprandial period or to augment the vasodilatory response to acute exercise are warranted and would have practical implications. In addition, future research should determine whether the effects of ingestion of whey-derived extracts can be augmented with higher or repeated doses throughout the day. Blockade studies are needed to further elucidate the precise mechanism(s)/pathway(s) by which novel whey-derived extracts exert their effects on the cardiovascular system.

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