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Effects of whey protein on skeletal muscle microvascular and mitochondrial plasticity following 10 weeks of exercise training in men with type 2 diabetes

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Abstract: Skeletal muscle microvascular dysfunction and mitochondrial rarefaction feature in type 2 diabetes mellitus (T2DM) linked to low tissue glucose disposal rate (GDR). Exercise training and milk protein supplementation independently promote microvascular and metabolic plasticity in muscle associated with improved nutrient delivery, but combined effects are unknown. In a randomised-controlled trial, 24 men (55.6 y, SD 5.7) with T2DM ingested whey protein drinks (protein/carbohydrate/fat: 20/10/3 g; WHEY) or placebo (carbohydrate/fat: 30/3 g; CON) before/after 45 mixed-mode intense exercise sessions over 10 weeks, to study effects on insulin-stimulated (hyperinsulinemic clamp) skeletal-muscle microvascular blood flow (mBF) and perfusion (near-infrared spectroscopy), and histological, genetic, and biochemical markers (biopsy) of microvascular and mitochondrial plasticity. WHEY enhanced insulin-stimulated perfusion (WHEY-CON 5.6%; 90% CI −0.1, 11.3), while mBF was not altered (3.5%; −17.5, 24.5); perfusion, but not mBF, associated (regression) with increased GDR. Exercise training increased mitochondrial (range of means: 40%–90%) and lipid density (20%–30%), enzyme activity (20%–70%), capillary:fibre ratio (∼25%), and lowered systolic (∼4%) and diastolic (4%–5%) blood pressure, but without WHEY effects. WHEY dampened *PGC1α* −2.9% (90% compatibility interval: −5.7, −0.2) and *NOS3* −6.4% (−1.4, −0.2) expression, but other messenger RNA (mRNA) were unclear. Skeletal muscle microvascular and mitochondrial exercise adaptations were not accentuated by whey protein ingestion in men with T2DM. ANZCTR Registration Number: ACTRN12614001197628.

Novelty:

- Chronic whey ingestion in T2DM with exercise altered expression of several mitochondrial and angiogenic mRNA.
- Whey added no additional benefit to muscle microvascular or mitochondrial adaptations to exercise.
- Insulin-stimulated perfusion increased with whey but was without impact on glucose disposal.

Key words: insulin resistance, intense exercise, near-infrared spectroscopy, angiogenesis.

Résumé : Le dysfonctionnement microvasculaire du muscle squelettique et la raréfaction mitochondriale caractérisant le diabète de type 2 (« T2DM ») sont liés à un faible taux d'élimination du glucose tissulaire (« GDR »). L'entraînement physique et la supplémentation en protéines du lait favorisent indépendamment la plasticité microvasculaire et métabolique dans le muscle; cette plasticité est associée à une amélioration de l'apport de nutriments, mais les effets combinés sont inconnus. Dans un essai contrôlé randomisé, 24 hommes (55,6 ans, SD 5,7) aux prises avec le T2DM consomment des boissons protéinées de lactosérum (protéines / glucides / lipides: 20/10/3 g; « WHEY ») ou un placebo (glucides / lipides: 30/3 g; « CON ») avant / après 45 séances d'exercice intense en mode mixte sur 10 semaines, et ce, pour examiner les effets sur le flux sanguin microvasculaire (« mBF ») et la perfusion (spectroscopie proche infrarouge) stimulés par l'insuline (clamp hyperinsulinémique), des variables histologiques, génétiques et des marqueurs biochimiques (biopsie) de la plasticité microvasculaire et mitochondriale. WHEY améliore la perfusion stimulée par l'insuline (WHEY-CON 5,6 %; IC 90 % −0,1, 11,3), tandis que le mBF n'est pas modifié (3,5 %; −17,5, 24,5); la perfusion, mais pas le mBF, est associée (régression) à une augmentation du GDR. L'entraînement à l'exercice augmente la densité mitochondriale (gamme de moyennes: 40-90 %) et lipidique (20−30 %), l'activité enzymatique (20−70 %), le ratio capillaire: fibre (∼25 %) et diminue les pressions systolique (∼4 %) et diastolique (4−5 %), mais sans effets de WHEY. WHEY amortit l'expression de *PGC1α* −2,9 % (intervalle de compatibilité de 90 % : −5,7, −0,2) et *NOS3* −6,4 % (−1,4, −0,2), mais les autres ARN messager (ARNm) ne sont pas clairs. Les adaptations microvasculaires et mitochondriales des muscles squelettiques causées par l'entraînement physique ne sont pas accentuées par

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la consommation de protéines de lactosérum chez les hommes aux prises avec le T2DM. Numéro d'enregistrement ANXCTR : ACTRN12614001197628. [Traduit par la Rédaction]

Les nouveautés:

- La consommation prolongée de lactosérum en présence de T2DM combinée à l'entraînement physique modifie l'expression de plusieurs ARNm mitochondriaux et angiogéniques.
- Le lactosérum n'ajoute aucun avantage supplémentaire aux adaptations microvasculaires ou mitochondriales musculaires à l'exercice physique.
- La perfusion stimulée par l'insuline augmente avec le lactosérum mais n'a pas d'impact sur l'élimination du glucose.

Mots-clés : résistance à l'insuline, exercice intense, spectroscopie proche infrarouge, angiogenèse.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterised by diminished glucose disposal at skeletal muscle secondary to insulin resistance within one or more sites, including the microvasculature and the myofiber. At the vascular endothelium, insulin stimulates nitric oxide synthetase (eNOS) signalling to increase nitric oxide (NO) formation promoting vascular relaxation, skeletal muscle blood flow and capillary recruitment (Barrett et al. 2011; Vincent et al. 2004; Zhang et al. 2004). The elevation of blood kinetics through the microvasculature increases both the delivery of insulin to the endothelial receptors for insulin-regulated insulin transport, and the delivery of glucose for transporter-mediated diffusion across the sarcoplasmic membrane (Barrett et al. 2011). In T2DM, however, normal vascular endothelial NO formation and vascular relaxation is impaired (Laakso et al. 1992; Williams et al. 1996) and the insulin-stimulated upregulation of muscle blood flow (mBF) and volume is diminished (Clerk et al. 2007; Padilla et al. 2006; St-Pierre et al. 2010). Vascular responses account for up to 40% of the increase in glucose disposal after eating (Fugmann et al. 2003) and the restoration of vascular sensitivity to insulin may be critical to the enhancement of glycaemic control in people with T2DM.

Mitochondrial rarefaction is another feature of skeletal muscle in people with T2DM that has been associated with hyperglycaemia and low glucose disposal (Hsieh et al. 2011; Kelley et al. 2002; Meex et al. 2010; Mogensen et al. 2007). It has been posited that reduced mitochondrial density lowers metabolic activity in muscle cells, including the normal oxidation of lipid species. Elevated levels of lipid, lipid-derived substrates (Bergman et al. 2012; Boon et al. 2013; Haus et al. 2009; Kuzmenko and Klimentyeva 2016) and reactive oxygen species (Schrauwen et al. 2010; Wohaieb and Godin 1987) have been observed in the skeletal muscle of individuals with T2DM and shown to interfere with insulin signal transduction (Anderson et al. 2009; Boon et al. 2013; Tirosh et al. 1999). Meanwhile, mitochondrial expansion after exercise training has been positively associated with improvements in glucose disposal rate (GDR) (O'Gorman et al. 2006) and interventions that promote mitochondrial expansion and fat oxidation capacity may be of therapeutic benefit for improving glycaemic control in populations with T2DM (Pruchnic et al. 2004).

Chronic dairy-based dietary protein supplementation during exercise training has shown promise as an adjunct intervention that may complement or accentuate improvements in vascular and mitochondrial function in people with metabolic syndrome or T2DM. In hypertensive rats, 6 weeks of casein hydrolysate supplementation increased the concentration of arterial eNOS and had anti-hypertensive effects, relative to tap water control (Sánchez et al. 2011). Relative to isoenergetic sodium caseinate, 8 weeks of casein hydrolysate with lactotripeptides ingestion twice daily by postmenopausal women during 8 weeks of aerobic exercise training produced significant improvements in arterial flow mediated dilation, a NO-driven measure of vascular relaxation (Yoshizawa et al. 2010). Transcriptomic analyses of skeletal muscle in response to whey-casein protein ingested after endurance cycling revealed over-expression of genes associated with microvascular and neuronal developmental processes important for increased tissue capillary growth, relative to the ingestion of equicaloric ingestion carbohydrate and fat (Rowlands et al. 2011). Similarly, genes associated with cell energy status and metabolism were upregulated in skeletal muscle after endurance cycling in the presence of whey-casein protein, carbohydrate and fat supplementation vs carbohydrate and fat alone (Rowlands et al. 2011). At the level of protein syntheses, another key part of the molecular regulation of cellular phenotype, most acute studies show evidence for substantially increased skeletal-muscle myofibrillar protein synthesis from whey protein (or whey and leucine) ingestion associated with exercise (Breen et al. 2011; Churchward-Venne et al. 2019; Coffey et al. 2011; Rowlands et al. 2015), but effects on mitochondrial fraction are largely neutral (Breen et al. 2011; Churchward-Venne et al. 2019; Coffey et al. 2011). There is some evidence, albeit mixed, however, to suggest that chronic whey protein added to a carbohydrate consumed during and after endurance exercise may accentuate mitochondrial adaptation; for example, vs isocaloric non-protein controls, 2 week supplementation in trained men increased skeletal muscle peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (Hill et al. 2013), 6 weeks of a milk protein supplement increased maximal oxygen uptake ($\dot{V}O_{2max}$), but without change on accumulated muscle protein in aged men (Robinson et al. 2011), and 6-weeks of protein plus carbohydrate supplementation produced significant increases in some but not all mitochondrial proteins measured (Hansen et al. 2020).

A whey-protein rich drink ingested proximal to exercise is a practical intervention with some potential for benefit and almost no risk of harm. Therefore, the purpose of this study was to determine if whey protein supplementation coupled with 10 weeks of exercise training could impact on messenger RNA (mRNA) measures of microvascular and mitochondrial development and insulin-stimulated hemodynamic phenotype (flow and volume), and on mitochondrial expansion in men with T2DM. We hypothesised that the repeated nutritional stimuli (45 exposures) would provide a chronic transcriptional-protein expression response (Hawley et al. 2018) to accentuate plasticity within the skeletal-muscle microvascular phenotype and myocellular leading to elevated markers of mitochondrial and metabolic adaptation associated with exercise-training mediated increases in glucose disposal. To conduct this work, we extended our investigation of skeletal muscle tissue from a recent report on glycaemic effects (Gaffney et al. 2018).

2. Materials and methods

2.1. Participants

Men with T2DM ($n = 24$), aged 40–65 y, body mass index < 40 kg·m^{-2}, not requiring insulin therapy, and not meeting the American College of Sports Medicine guidelines for exercise for T2DM (Colberg et al. 2010) were recruited from medical centres in Wellington, New Zealand, between January 2014 and May 2015. Participants were screened for precluding health complications as discussed in our previous report (Gaffney et al. 2018). Three participants were excluded due to exercise >150 min·wk^{-1} ($n = 1$), irregular cardiac rhythm ($n = 1$), technical difficulty during insulin clamp

($n = 1$). Ethics was approved by the Northern B Health and Disability Ethics Committee, Ministry of Health, Wellington, NZ (13/NTB/69). Participants provided written informed consent.

2.2. Study design

The design was a double-blind, randomised (Research Randomizer, Version 4.0, http://www.randomizer.org), placebo-controlled trial (http://www.anzctr.org.au/, ANZCTR Registration No. ACTRN12613000340730) summarised in Supplementary Fig. S1.[1] At early stages of data collection, the original intended third group — whey without exercise training — was removed from the study design because recruited eligible participants declined to participate if not randomised to an exercise group, creating sampling bias. Thereafter, participants were randomly allocated from a sequentially numbered envelope based on derived randomised sequence and presented by a third party, into a whey protein-carbohydrate supplement (WHEY) or an isocaloric carbohydrate placebo (CON) group. The intervention occurred during 10 weeks of mixed-mode intense exercise training as previously described (Gaffney et al. 2018) conducted at university research laboratories and gymnasium. The insulin clamps were conducted in a research laboratory at Wellington Hospital. Briefly, the exercise regimen included 3 days of intensive interval cycling (20 min) with 2 additional intensive interval resistance training sessions (20 min) each week for 10 weeks (total 45 sessions). A chocolate-flavoured whey protein isolate (WPI-895; Fonterra, Auckland, New Zealand) beverage (20 g protein/10 g carbohydrate/3 g milk fat) or an identically flavoured but non-protein-formulated isocaloric beverage (30 g carbohydrate/3 g milk fat) was consumed immediately before and after each exercise session. Each drink contained 175 kcal (731 kJ). To reduce hunger and provide opportunity for a clear peri-training whey compared with carbohydrate consumption effect to be observed, each participant consumed a low-protein snack bar (Nature Valley; General Mills, Auckland, NZ) 1 h after exercise and resumed normal eating habits after 2 h. Participants were familiarised with all testing procedures except the euglycemic insulin clamp before baseline testing. Cardiac screening via ECG was performed at familiarisation during a peak oxygen consumption cycling test. Baseline testing occurred 5–10 d before commencement of the intervention with post-testing 2 d after 45 exercise sessions. The post-glucose clamp was performed 48 h after maximal cycling and strength tests to provide a washout period that would allow for the bulk of the acute effects of intense exercise on glycaemia to return to pre-exercise levels without inducing a period of deconditioning (Gaffney et al. 2018).

2.3. Microcirculation

Near infrared spectroscopy (NIRS) was used to assess changes in mBF and perfusion (total blood volume) at the vastus lateralis (VL) muscle using previously validated methods (Gaffney et al. 2018). Briefly, a NIRS probe was secured over the belly of the VL muscle measured two-thirds from the top of the muscle and parallel to the muscle fibres. VL muscle and subcutaneous adipose tissue thickness was determined using B-mode ultrasound (Terason; United Medical Instruments Inc., San Jose, Calif., USA) to ensure that photon projection was located within skeletal muscle and not subcutaneous tissue; all participants had subcutaneous adipose tissue thickness <20 mm required for reliable depth penetration of the NIRS light into skeletal muscle. Wavelengths were emitted from LEDs at 760 and 850 nm and collected at 10 Hz to detect relative changes in the concentration of oxygenated haemoglobin [HbO_2] and deoxygenated haemoglobin [HHb], respectively, as well as the haemoglobin concentration in the total blood volume ([tHb] = [HbO_2] + [HHb]). Blood volume was determined from the average total haemoglobin ([tHb]) concentration ($\mu mol \cdot L^{-1}$) over 5 min, at rest after lying supine for 45 min before (basal), then during insulin infusion (insulin-stimulated) and expressed as a surrogate measure of capillary recruitment. mBF was determined from the slope of the [tHb] signal during a 15-second venous cuff occlusion (70–80 mm Hg), and converted to $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ using the following equation (Beekvelt et al. 2001):

$$mBF = \frac{\Delta[tHb] \cdot 60}{([Hb] \cdot 1000)/4} \cdot 100$$

where [Hb] was determined from a resting blood draw, and Δ[tHb] averaged over 5 measurements taken 1 min apart.

2.4. Muscle sample collection

Muscle biopsies were obtained from the VL muscle under local anaesthesia using a Bergstrom needle (Bergstrom 1975) with suction. Muscle samples were immediately freed from blood, visible fat, and connective tissue. 5 mg of tissue was placed in Karnovsky's fix (Sheehan and Hrapchak 1980) for 2 hours and then stabilised in a sodium cacodylate buffer solution for electron microscopy analysis. The remaining tissue ~30 mg was immediately frozen in liquid nitrogen after extraction, then transferred to cryovials for storage at −80 °C.

2.5. Mitochondrial, capillary and lipid density

Mitochondrial and lipid density within the intermyofibrillar region of myocytes was assessed via transmission electron microscopy ×5800 (Philips CM100, Philips Electron Optics, Eindhoven, the Netherlands) using previously described methods (Rowlands et al. 2014). A minimum of 10 and up to 14 intermyofibrillar images of a muscle cell were taken for each sample, based on convergence (no further reduction) of the coefficient of variation (CV) for the dependent variables derived from analysis of 8 randomly selected samples, occurring on average with 10 samples, which concurred with a previously reported analysis approach utilising 8 images per region of interest (Samjoo et al. 2013). Mitochondrial and lipid density for each component were determined using Image J software (version 1.48v, National Institutes of Health, Bethesda, Md., USA) by manually tracing only clearly discernible outlines of mitochondria and lipid droplets on each image using a graphic tablet (MP1060-HA60, Monoprice, Calif., USA). Density was quantified as the average percentage of traced pixels to the number of pixels within the whole of each image.

Capillarisation was determined from a section of muscle tissue selected using a dissecting microscope and placed in cassettes in a Tissue Tek VIP 5 processor overnight. The tissue was orientated into cross sections, embedded and cut into 4-micron sections. Sections were collected on adhesive slides and dried for 1 h at 60 °C. Sections were stained with Periodic Acid Schiff. Capillarisation was expressed as the capillary to myofiber ratio (C/F ratio). Capillary counts were taken from sections of 100–150 fibres using light microscopy and digital photos taken at ×400 magnification; recent analysis suggests at least 50 fibres per fibre type is ideal for C/F quantification (Nederveen et al. 2020).

2.6. Citrate synthetase (CS) and cytochrome c oxidase (COX) analysis

Mitochondrial enzyme concentration for CS and COX was inferred from the rates of enzymatic activity ($\mu mol \cdot mL^{-1} \cdot min^{-1}$) derived from colorimetric assay on a microplate platform, normalised to total protein using BCA protein assay (Rowlands et al. 2014, 2011).

2.7. mRNA expression analysis

Total RNA was extracted (~10 mg muscle tissue) using the Norgren RNA/DNA/Protein Purification Plus Kit (#47700) as per the manufacturer's protocol. RNA quantity and quality were assessed

[1]Supplementary data are available with the article at https://doi.org/10.1139/apnm-2020-0943.

using a Nanodrop and Agilent Bioanalyser respectively. Total RNA (48 ng) was reversed transcribed (SuperScript III VILO cDNA Synthesis Kit, Life Technologies) as per the manufacturer. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate per sample using 2 μL 1/2 dilution complementary DNA (cDNA), and TaqMan gene expression assays, vascular endothelial derived growth factor A (*VEGFA*) (Hs00900055_m1), vascular endothelial derived growth factor receptor 2 (*VEGFR2*) (Hs04959920_s1), endothelial nitric oxide synthase (*NOS3*) (Hs01574659_m1) *PGC1α* (Hs01016719_m1), *CS* (Hs02574374_s1), nuclear respiratory factor 1 (*NRF1*) (Hs01031046_m1), glucose transporter 4 (*SLC2A4*) (Hs00168966_m1), insulin-like growth factor 2 (*IGF2*) (Hs04188276_m1) and DNA methyltransferase 3B (*DNAMT3B*) (Hs00171876_m1). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Hs04420566_g1) and β_2 microglobulin (*B2M*) (Hs00984230_m1) were used as endogenous controls. Statistical differences were all *unclear* within or between groups for these 2 controls: summarised as *gene*, group Post-Pre contrast score, (90% compatibility interval, CI): *GAPDH,* CON 2.4% (−2.4, 7.1), WHEY −0.6% (−3.9, 2.6), WHEY-CON −3.0% (−8.3, 2.3); *B2M*, CON 0.7% (−2.7, 4.1), WHEY −2.4% (−5.7, 0.8), WHEY-CON −3.2 (−7.2, 0.9). Accordingly, the combined mean expression (Ct) for GAPDH and B2M was used to normalise target mRNA expression using the ΔCt method: $\Delta Ct = Ct_{targetmRNA} - meanCt_{controls}$.

2.8. GDR

GDR for each individual at Week 0 and Week 10 was determined via a modified hyperinsulinemic-euglycemic clamp as previously described (Matsuda and DeFronzo 1999). Briefly, participants received a priming insulin dose of 160 mU·(m^2 body mass·min)$^{-1}$ for 4 min and 80 mU·(m^2 body mass·min)$^{-1}$ for 3 min, after which the dosage was reduced to 40 mU·(m^2 body mass·min)$^{-1}$ for the remainder of the clamp. A 25% glucose infusion was initiated at 15 min or sooner if fasting blood glucose levels were below 6.5 mmol·L^{-1} and adjusted after 5-minute blood glucose readings until stabilised at 5 mmol·L^{-1}. GDR was calculated from the average rate of glucose infused (mg·kg^{-1}·min^{-1}) during a 60-minute stabilisation.

2.9. Sample size

Sample size estimation was based upon the limited a priori available test−retest measures for GDR (CV 3.74%) reported by DeFronzo et al. (1979) and sample size estimations for magnitude-based decisions for parallel-groups controlled trials and minimum-effects testing framework, with 0.05 and 0.25 maximal clinical type-1 and type-2 error rates (Hopkins and Batterham 2016; Hopkins et al. 2009) and the value 5.4% for smallest clinically-meaningful effect based on 12-week metformin with basal between-SD of 11.2% (Derosa et al. 2009). These estimates provided power for a most likely clear outcome from an anticipated comparable effect size of a statistically significant 23% increase in GDR response in T2DM patients after 12 weeks standard pioglitazone+metformin intervention (Derosa et al. 2009).

2.10. Statistical analysis

The effect of treatment and time on all dependent variables was estimated from mixed models (Proc Mixed, SAS Version 9.1; SAS Institute, Cary, N.C., USA) and reported as the mean post-pre change covariate adjusted for the baseline value. Microvascular phenotype, immunoflurochemistry, enzyme activity and gene expression data were normalised to percent change following 100-times natural log transformation. Linear regression was used to determine within- and between-group associations between the standardised week 10–0 change score for the predictor variables (*x*-axis) mitochondrial density, insulin-stimulated blood flow and perfusion, on the dependent variable (*y*-axis) week 10–0 change in GDR. Associations on the dependent were reported relative to standardised magnitudes of increase of the value of the predictor, within a relevant and convenient scale covering small through large standardised effect size scenarios, presented as 0.5, 1.0 and 2.0 SD (Hopkins et al. 2009).

Uncertainty was presented as 90% CI (Gelman and Greenland 2019). In keeping with recent calls to advance statistical analysis and reporting (Amrhein et al. 2019), inference was based on probabilistic decisions about true (large sample) magnitudes based on two 1-sided hypothesis tests (TOST) of the smallest important effect (SIE) (Hopkins et al. 2009). The approach sits within the inferential family of equivalence, non-inferiority and minimal effects or superiority testing (Dunnett and Gent 1996; Piaggio et al. 2006; US Department of Health and Human Services Food and Drug Administration 2016). The threshold for SIE was 5.4% for GDR and the Cohen *d* smallest standardised difference (0.2 SD) for all mechanistic parameters, with the rationale being that current exploration of microvascular hemodynamic effects with NIRS on glucose disposal in humans with T2DM is novel and we have no a priori information on physiological effect sizes. Under this scheme, the *p* value for rejecting a hypothesis of a given magnitude was the area of the sampling *t* distribution of

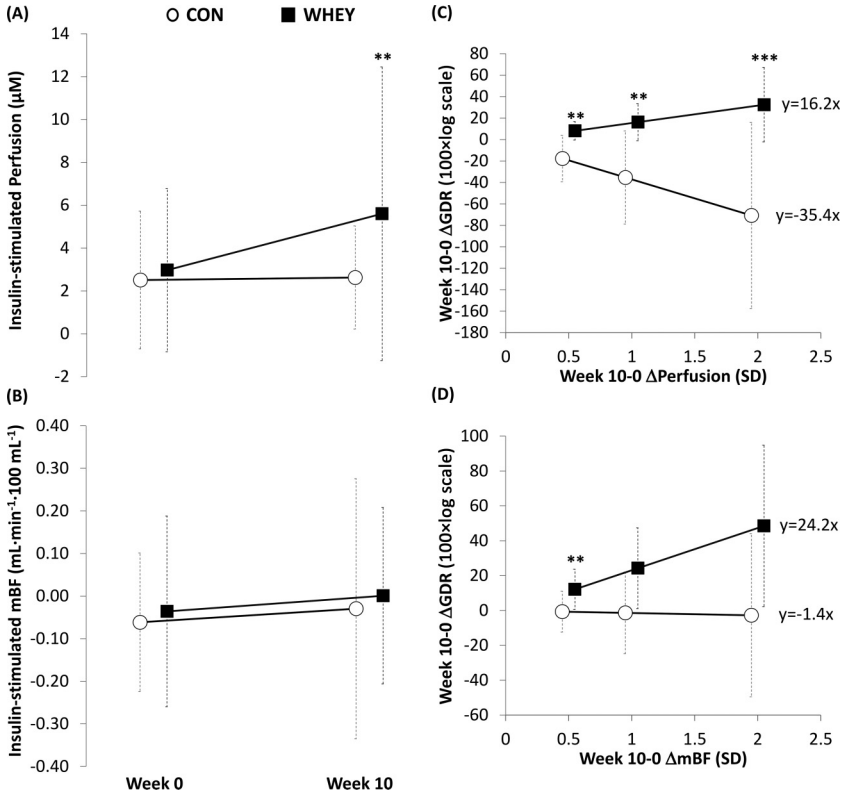
Table 1. Group characteristics at baseline prior to initiation of treatment.

Parameter	CON, *n* = 12	WHEY, *n* = 12
Age, y	57.8 (5.2)	53.5 (5.6)
Height, cm	174.6 (7.1)	177.1 (8.7)
Weight, kg	91.9 (15.5)	92.8 (11.0)
BMI, kg·m^{-2}	30.1 (4.9)	29.6 (2.7)
$\dot{V}O_{2peak}$, mL·kg^{-1}·min^{-1}	26.9 (10.2)	28.7 (4.9)
FBG, mmol·L^{-1}	9.4 (2.9)	10.2 (3.6)
Systolic blood pressure, mm Hg	129 (12)	134 (14)
Diastolic blood pressure, mm Hg	78 (7)	85 (8)
Mitochondrial density, pixels/image × 1000	29.8 (12.8)	30.2 (11.6)
Lipid density, pixels/image × 1000	3.98 (3.75)	5.44 (5.46)
Capillary to fibre ratio	0.7 (0.3)	0.7 (0.1)
Citrate synthase, μmol·mL^{-1}·min^{-1}	0.13 (0.07)	0.13 (0.09)
COX, μmol·mL^{-1}·min^{-1}	0.11 (0.030	0.09 (0.03)
Basal VL microvascular blood flow, mL·min^{-1}·100 mL^{-1}	0.73 (0.20)	0.63 (0.25)
Basal VL microvascular blood volume, μmol·L^{-1}	59.0 (11.5)	59.3 (15.6)
Insulin-stimulated VL microvascular blood flow, mL·min^{-1}·100 mL^{-1}	−0.062 (0.163)	−0.036 (0.22)
Insulin-stimulated VL microvascular blood volume, μmol·L^{-1}	2.5 (3.2)	3.0 (3.8)
VEGFA, ΔCt[b]	25.5 (0.3)	26.7 (0.7)
VEGFR2, ΔCt[b]	31.0 (0.7)	32.1 (0.8)
NOS3, ΔCt[b]	33.1 (0.7)	35.0 (1.0)
PGC1α, ΔCt[b]	28.0 (0.6)	29.1 (0.7)
CS, ΔCt[b]	24.7 (0.6)	25.9 (1.0)
NRF1, ΔCt[b]	27.7 (0.7)	29.2 (1.1)
SLC2A4, ΔCt[b]	26.1 (0.5)	27.3 (0.8)
IGF2, ΔCt[b]	31.9 (1.0)	32.9 (1.0)
DNAMT3B, ΔCt[b]	34.8 (1.1)	36.2 (1.3)

Note: Data are raw mean (SD). Basal (week 0) mean and SD for microvascular blood flow and perfusion are in Fig. 1 and lipid and mitochondrial density, and capillary to fibre ratio are in Fig. 2. BMI, body mass index; CON, supplement with 0 g whey protein; COX, cytochrome c oxidase; *CS*, citrate synthase; *DNAMT3B*, DNA methyltransferase 3B; FBG, fasting blood glucose; *IGF2*, insulin-like growth factor 2; mRNA, messenger RNA; *NOS3*, endothelial nitric oxide synthase; *NRF1*, nuclear respiratory factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *SLC2A4*, glucose transporter 4; *VEGFA*, vascular endothelial derived growth factor A; *VEGFR2*, vascular endothelial derived growth factor receptor 2; VL, vastus lateralis; $\dot{V}O_{2peak}$, peak oxygen uptake; WHEY supplement with 20 g whey protein.

[b]Raw mRNA data are baseline ΔCt scores normalised by division against the average of the 2 housekeeper gene expression (see Materials and methods).

Fig. 1. Insulin-stimulated (A) perfusion and (B) microvascular blood flow (mBF), and the regression analysis of 10-week change in (C) insulin-stimulated perfusion and (D) insulin-stimulated mBF against change in glucose disposal rate (GDR) in response to 10-weeks of whey protein supplementation coupled to exercise training. Data are raw means and SD for panels A and B and least-squares mean estimates and 90% compatibility interval derived from the statistical regression model for panels C and D. For panel D, the effector data (*y*-axis) is the 100 × log-transformed change in glucose disposal rate, with the outcome point data shown at 0.5, 1.0 and 2.0 SD of the predictor. The regression equation for effect on *y* at 1 SD of *x* for each condition, and the WHEY-CON is shown within the figure; the formula to convert *y* from 100 × log-transformed change to percent change is *y* (%) = 100 × [exp (*y* / 100) – 1]. The TOST probability defined the compatibility of which point estimate was at least greater than the SIE for GDR (5.4%) are as follows: 0.25–0.75, possible (*); 0.75–0.95, likely (**); 0.95–0.995, very likely (***); >0.995, almost certain (****). CON, supplement with 0 g whey protein; TOST, two 1-sided hypothesis tests; WHEY supplement with 20 g whey protein.



the effect statistic. Hypotheses of substantial decrease and increase (i.e., >< SIE) were rejected if their respective *p* values were less than 0.05. If 1 hypothesis was rejected, the *p* value for the other hypothesis was interpreted as evidence for the alternative hypothesis, since the *p*-value corresponds to the posterior probability of the magnitude of the true effect in a reference Bayesian analysis with a minimally informative prior (Burton 1994; Burton et al. 1998; Shakespeare et al. 2001). The *p* value was reported in reference Bayesian terms for strength of evidence, or compatibility with rejection: 0.25–0.75, *possible*; 0.75–0.95, *likely*; 0.95–0.995, *very likely*; >0.995, *almost certain* (Albers et al. 2018; Hopkins 2020; Hopkins et al. 2009; Mastrandrea et al. 2010, Shakespeare et al. 2001). If neither hypothesis was rejected, the magnitude of the effect was considered *unclear*. To reduce inflation of error arising from the large number of effects investigated, effects were considered *decisive* with more conservative *p*-value thresholds ($p < 0.01$ for a substantial decrease or increase).

3. Results

Group characteristics at baseline are in Table 1. GDR outcomes were published earlier (Gaffney et al. 2018). Raw mean (SD) baseline and post-intervention scores were 2.1 mg·kg^{-1}·min^{-1} (1.4) and 2.5 mg·kg^{-1}·min^{-1} (1.6) in WHEY and 1.9 mg·kg^{-1}·min^{-1} (0.8) and 2.5 mg·kg^{-1}·min^{-1} (1.2) in CON, respectively. The WHEY-CON post-pre effect on GDR after analysis of log-transformed data was 2.2% (−28.0, 44.8) (Gaffney et al. 2018). At week 10 in the WHEY and CON groups, systolic blood pressures were lowered from baseline (Table 1) to 126 mm Hg (SD 9) and 127 mm Hg (13), and diastolic lowered to 76 mm Hg (5) and 80 mm Hg (7), respectively. The within-group Week 10–0 decreases were substantial: systolic WHEY −4% (90% CI −8, −1), CON −4% (−8, 0); diastolic 4% (90% CI −9, −1), CON −5% (−9, −1), but the WHEY-CON effects were unclear at 0.4% (90% CI −5.9, 5.3) and 1.0% (−5.3, 7.7), for systolic and diastolic pressures, respectively.

3.1. Microcirculation

Basal and insulin-stimulated microvascular perfusion (total mBV) increased in the WHEY group following the 10-week exercise but not in the CON group; the insulin-stimulated WHEY-CON effect on perfusion was likely compatible with a substantial standardised effect size (Fig. 1A;. Table 2). In contrast, basal and insulin-stimulated mBF was not clearly affected by WHEY (Fig. 1B; Table 2).

The mechanistic regression associations between the 10-week change in perfusion and mBF vs GDR are shown in Fig. 1C–1D. The mean regression slope effects (slope β) of 1 SD change in the predictor on GDR are shown within the figures. For the WHEY-CON slope contrast, the regression association was very likely compatible with clear clinically meaningful effect size

Table 2. Basal and insulin-stimulated changes in skeletal muscle microvascular blood flow and perfusion in response to 10 weeks of whey protein supplementation coupled to exercise in men with type 2 diabetes.

	Raw mean change score (SD)[a]		Within group baseline adjusted estimate (90%CI)[b]		Baseline adjusted outcome for WHEY-CON	
Treatment contrast	CON	WHEY	CON	WHEY	Estimate (90%CI)[b,c]	Probability P_+/P_-
Perfusion						
	Units: $\mu mol \cdot L^{-1}$		Units: % Change		Units: % Change	
Basal	1.32 (9.52)	4.31 (7.38)	0.3 (–8.1, 8.7)	6.1 (0.2, 12.1)	5.8 (–4.1, 15.7)	0.57/0.039*
Insulin-stimulated	0.12 (2.72)	2.63 (7.70)	0.9 (–1.2, 3.1)	6.5 (1.1, 11.9)	5.6 (–0.1, 11.3)	0.91/0.031**
Microvascular blood flow						
	Units: $mL \cdot min^{-1} \cdot 100\ mL^{-1}$		Units: % Change		Units: % Change	
Basal	0.018 (0.355)	0.082 (0.210)	2.8 (–16.2, 21.9)	10.3 (–2.4, 23.0)	7.5 (–14.5, 29.4)	0.54/0.15
Insulin-stimulated	0.032 (0.294)	0.037 (0.274)	2.4 (–14.5, 19.3)	5.9 (–8.4, 20.2)	3.5 (–17.5, 24.5)	0.43/0.23

Note: CI, compliance interval; CON, supplement with 0 g whey protein; P_+/P_-, probability from the TOST; SIE, smallest important effect; TOST, two 1-sided hypothesis tests; WHEY supplement with 20 g whey protein.

[a]Post−pre unadjusted raw change scores.

[b]Back log-transformed estimates expressed as percent derived from the mixed model analysis adjusted for baseline covariate.

[c]The TOST Probability defined compatibility that a contrast was at least >SIE: 25–0.75, *possible* (*); 0.75–0.95, *likely* (**); 0.95–0.995, *very likely* (***); >0.995, *almost certain* (****). *Unclear* (unmarked) refers to outcomes where $p > 0.05$ for both increase and decrease relative to the SIE.

(i.e., >5.4% improvement in GDR) at 2 SD (β on GDR 181% increase in GDR at 2 SD increase in perfusion; 90% CI 10, 615; P_+/P_- 0.95/0.030) and likely compatible at 0.5SD (29%; 90% CI 2.5, 64; P_+/P_- 0.93/0.018) and 1 SD (68%; 5, 167; P_+/P_- 0.93/0.018) increases in perfusion, respectively. To place in context with basal perfusion (Table 1), the smallest clinical change after WHEY-CON is the value for smallest clinical change (5.4%)/100 × Ln (GDR) at 1 SD perfusion, times 1 SD value for change in perfusion (5.8 $\mu mol \cdot L^{-1}$, Fig. 1C), which yields a value of 0.61 $\mu mol \cdot L^{-1}$ increase in perfusion; for further reference, at the clinical effect size of 23% against pioglitazone+metformin intervention (Derosa et al. 2009), the value for perfusion is 2.6 $\mu mol \cdot L^{-1}$. With respect to the mBF-GDR regression association, while there was a likely compatible WHEY association with GDR (Fig. 1D), only at 0.5 SD was the WHEY-CON contrast evident of a substantial effect size (β on GDR 12.9%; 0.6%, 26.7%; P_+/P_- 0.91/0.021); larger effects of mBF on GDR association were unclear.

3.2. Densitometry, gene expression and enzyme activity

Skeletal muscle mitochondrial, lipid, and capillary density all increased in response to the 10 weeks exercise training, but there was no clear effect of WHEY (Fig. 2A–2C; Table 3). However, WHEY protein attenuated the decline in mitochondrial density associated with GDR (Fig. 1D). The pattern of *VEGFA*, *VEGFR2,* and *NOS3* gene expression varied between conditions in response to treatment; there were some reductions in mRNA expression level in response to WHEY, including a 6.4% mean reduction in basal *NOS3* expression (Table 3). Mitochondrial biogenic co-factor *PGC1α* and *CS* expression also varied in response to exercise but expression of both decreased 2.9% and 2.5%, respectively with WHEY, with the effect on *PGC1α* expression compatible with substantial reduction relative to the 0.2 SD Cohen *d* threshold criteria (Table 3).

4. Discussion

The principle observations from the current study was increased insulin-stimulated microvascular blood perfusion associated by linear regression with a clinically meaningful increase in GDR in response to whey-protein supplementation proximal to exercise. However, in the majority of the other outcomes, including microvascular blood flow and all other measures of microvascular and mitochondrial plasticity, observations were largely inconclusive or unsupportive of favourable benefits of whey protein. While most data are non-supportive, the perfusion association suggests that further exploration of the microvascular response to chronic dietary protein supplementation coupled with exercise and insulin resistance is warranted because perfusion controls muscle glucose uptake by increasing glucose dispersion (McClatchey et al. 2019).

Investigation in healthy populations suggest that capillary recruitment and therefore perfusion is the primary mechanism regulating glucose disposal (Coggins et al. 2001; Eggleston et al. 2007), which is supported by our current results. These observations present the question of regulation of modified nutrition−exercise factors that regulate insulin-stimulated vascular hemodynamics. At the macrovascular level, chronic whey protein supplementation lowered systolic blood pressure and increased flow-mediated dilation in pre- and mild-hypertensive adults, who were overweight or obese (Yang et al. 2019; Fekete et al. 2016). Basal systolic and diastolic blood pressures were lowered on average by 4–5 mm Hg (4%–5%) following exercise in both groups but was not additionally enhanced by WHEY in the current cohort, suggesting that the lowering effects of exercise ameliorated any whey-protein effects or that, possibly, T2DM was a modifier. Previously, casein hydrolysate supplementation for 6 weeks increased eNOS expression and relaxation at the macrovascular level of the aortas in hypertensive rats (Sánchez et al. 2011). In healthy adults, twice daily supplementation (28 g) with whey or casein protein for 8 weeks significantly improved flow mediated dilation — a marker of eNOS driven vascular endothelial function (relaxation) — compared with a carbohydrate control (Fekete et al. 2016). Eight weeks of daily whey protein supplementation (2 × 28 g) lowers blood pressure and improves flow mediated dilation (Fekete et al. 2016). Owing to technical issues we had to discontinue our macrovascular methodology during the study (flow mediated dilation and arterial stiffness), and acknowledge that the aorta is regulated somewhat differently to human microvascular tissue, but we investigated whole-muscle angiogenic gene expression and capillary formation within the microvasculature expecting to see a difference associated with the microvascular phenotype, but while capillary density increased after exercise training, it was not clearly affected with WHEY; furthermore, the observed decreased basal whole-muscle *NOS3* and *VEGFA*/*R2* expression suggest lower basal molecular signalling for angiogenesis, although protein markers could have been more conclusively informative. While we cannot discount increased protein activity, our current data suggest a change in eNOS expression or capillary density did not underlie the WHEY-mediated mechanism for improved perfusion, and more investigation is required.

The current study showed noteworthy increases in mitochondrial density and enzyme activity in response to exercise training, consistent with a previous report (Little et al. 2010), and increased $\dot{V}O_{2max}$ (Gaffney et al. 2018). However, increased mitochondrial density was not clearly accentuated by whey protein

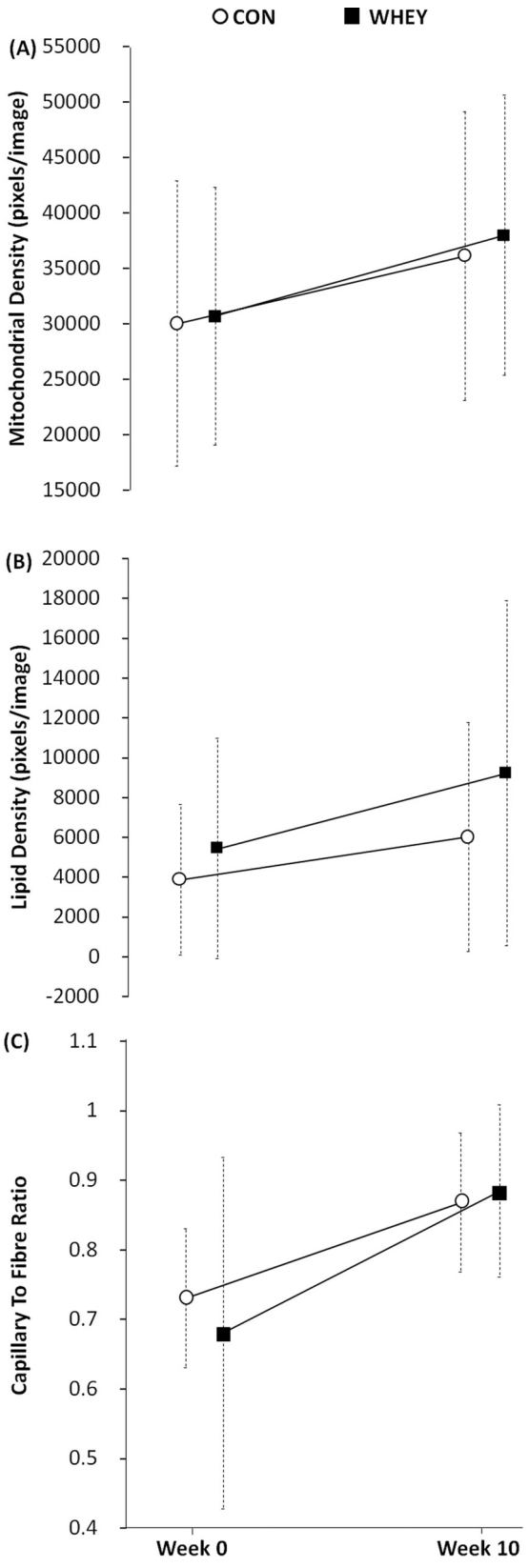


Fig. 2. Vastus lateralis muscle (A) mitochondrial density, (B) lipid droplet density, and (C) capillary to fibre ratio in response to 10 weeks of exercise and whey protein supplementation. Data are raw means and SD. There were no clear effects of treatment, therefore no statistical symbols are shown. CON, supplement with 0 g whey protein; WHEY supplement with 20 g whey protein.

supplementation. Previously, investigations of mitochondrial expansion have largely assessed short-term or signalling responses to combined protein-exercise treatments. Consumption of whey protein at 1.2 g·kg body weight^{-1} added to a sports drink daily for 2 weeks increased myocellular *PGC1α* expression 6 hours after a bout of cycling (Hill et al. 2013). In contrast, consuming whey protein and carbohydrate after a single bout of endurance cycling did not enhance mitochondrial synthetic rates compared with carbohydrate alone (Breen et al. 2011). In the current study, reductions in *PGC1a* and *CS* expression were observed, suggesting a possible dampening of signalling for mitochondrial biogenesis after whey protein supplementation. To compare with other groups, milk protein supplement (23 g) ingested after treadmill training 3 times per week for 6 weeks in previously sedentary older men significantly increased the improvement in training-mediated $\dot{V}O_{2max}$; no significant increase was seen in myocellular mitochondrial DNA concentration within the VL (Robinson et al. 2011). In a 10-week controlled trial in previous untrained younger individuals, protein supplementation elicited greater gains in $\dot{V}O_{2max}$ and stimulated lean mass accretion but did not improve skeletal muscle oxidative capacity and endurance performance (Knuiman et al. 2019). Taken together, our data suggest, and are supported by other cohort data, that there is currently no firm evidence that chronic milk protein supplementation combined with carbohydrate and fat energy accentuates $\dot{V}O_{2max}$ or mitochondrial development in previously sedentary adults with T2DM undertaking intense exercise training.

An interesting observation was that both groups showed an equivalent increase in lipid droplet density after 10 weeks. Low mitochondrial density has been previously linked to myocellular lipid accumulation and insulin resistance (Coen and Goodpaster 2012; Furler et al. 2001; Goodpaster et al. 2001; Petersen et al. 2004), with considerable speculation that elevated lipid leads to an increase in the formation of lipid substrates and pro-oxidative species that interfere with insulin sensitivity at the cell membrane surface. The increase in lipid density in the current study was not affiliated with detrimental glucose disposal, as both groups showed improvements in glucose disposal, fasting blood glucose and HOMA-IR (Gaffney et al. 2018). Lipid accretion has been considered a pro-adaptive response to endurance training in athletes (Goodpaster et al. 2001) and a recent investigation comparing myocellular lipid accretion after exercise in athletic, overweight and insulin resistant men found that insulin sensitivity was positively associated with lipid synthetic rates after exercise (Bergman et al. 2012). Taken together with the current findings, there is mounting evidence that lipid accumulation is not detrimental to glycaemic control in the exercise trained state. A limitation in the current study was that mitochondrial and lipid density were only assessed at the intramyofibrillar region of the harvested muscle cells and there has been some evidence that adaptations of this kind may be inversely produced at the subsarcolemmal regions of muscle cells (Nielsen et al. 2010). It was also raised in review that ceramide and diacylglycerol analysis may have been interesting. We decided against ceramide analysis due to cost and feasibility limitations in our hands, and due to remaining uncertainty as to the role with in skeletal muscle of these lipid metabolites in insulin resistance (Kitessa and Abeywardena 2016).

Table 3. Changes in molecular and histological measures of skeletal muscle microvascular and metabolic plasticity in response to 10 weeks of whey protein supplementation coupled to exercise in men with T2DM.

	Raw unit mean change score (SD)[a]		Within group adjusted estimate as % change (90%CI)[b]		Baseline adjusted outcome for as % change (90%CI)[b]	
Treatment outcome	CON	WHEY	CON	WHEY	WHEY-CON[c]	Probability P_+/P_-
Microvascular						
Capillary to fibre ratio	0.13 (0.29)	0.20 (0.29)	24.5 (−0.1, 55)	26.3 (1.9, 56.6)	1.5 (−25.3, 38)	0.41/0.33
VEGFA (ΔCt)[a]	0.22 (0.63)	−0.14 (0.40)	3.6 (−3.2, 10.3)	−1.3 (−5.0, 2.4)	−4.9 (−12.0, 2.3)	0.046/0.72*
VEGFR2 (ΔCt)[a]	−0.36 (0.43)	−0.78 (0.70)	−3.9 (−7.7, −0.1)	−7.6 (−10.7, −4.5)	−3.7 (−8.1, 0.8)	0.045/0.85**
NOS3 (ΔCt)[a]	0.26 (0.41)	−0.33 (0.58)	5.6 (1.6, 9.6)	−0.8 (−4.0, 2.3)	−6.4 (−1.4, −0.2)	0.007/0.97***
Mitochondria and metabolic						
Mitochondrial density (pixels/image ×1000)	5.6 (16.3)	8.6 (17.9)	22.9 (6.9, 41.4)	30.8 (12.1, 52.5)	6.4 (−12.4, 29.2)	0.47/0.08
Lipid density (pixels/image × 1000)	2.1 (2.8)	3.7 (3.7)	47.1 (−7.6, 134)	91,2 (39.6, 162)	30 (−23, 120)	0.72/0.06
CS activity (%)	47.3 (76.4)	54.9 (411.7)	67.3 (28.0, 118.7)	22.1 (−20.2, 86.8)	−27 (−55, 17.3)	0.11/0.35
COX activity (%)	30.1 (57.2)	40.8 (58.3)	29.9 (−6.9, 81.3)	31.5 (6.1, 63.0)	1.2 (−30.3, 47.1)	0.34/0.29
PGC1α (ΔCt)[a]	0.14 (0.35)	−0.15 (0.37)	1.5 (−0.7, 3.7)	−1.4 (−3.6, 0.7)	−2.9 (−5.7, −0.2)	0.008/0.81**
CS (ΔCt)[a]	0.14 (0.26)	−0.08 (0.26)	2.3 (−0.0, 4.6)	−0.2 (−4.3, 3.9)	−2.5 (−6.9, 1.9)	0.025/0.43*
NRF1 (ΔCt)[a]	0.71 (0.38)	0.33 (0.61)	8.1 (4.4, 11.8)	6.9 (3.7, 10.1)	−1.2 (−5.7, 3.2)	0.13/0.42
SLC2A4 (ΔCt)[a]	0.44 (0.36)	0.25 (0.41)	7.0 (2.9, 11.0)	6.1 (1.5, 10.7)	−0.9 (−6.5, 4.7)	0.23/0.42
IGF2 (ΔCt)[a]	−1.37 (0.73)	−1.14 (1.11)	−13.9 (−20.2, −7.6)	−11.6 (−18.3, −4.8)	2.3 (−6.0, 10.7)	0.58/0.22
DNAMT3B (ΔCt)[a]	−0.04 (0.74)	−0.21 (0.38)	−1.0 (−3.6, 1.7)	−0.7 (−2.5, 1.0)	0.2 (−2.6, 3.1)	0.42/0.31

Note: CI, compliance interval; CON, supplement with 0 g whey protein; COX, cytochrome c oxidase; *CS*, citrate synthase; *DNAMT3B*, DNA methyltransferase 3B; *IGF2*, insulin-like growth factor 2; mRNA, messenger RNA; *NOS3*, endothelial nitric oxide synthase; *NRF1*, nuclear respiratory factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; P_+/P_-, probability from the TOST; SIE, smallest important effect; *SLC2A4*, glucose transporter 4; TOST, two 1-sided hypothesis tests; *VEGFA*, vascular endothelial derived growth factor A; *VEGFR2*, vascular endothelial derived growth factor receptor 2; WHEY supplement with 20 g whey protein.

[a]Post−pre unadjusted raw change scores. Raw mRNA data are post−pre ΔCt scores normalised by division against the average of the 2 housekeeper gene expression (see Materials and methods).

[b]Back log-transformed estimates expressed as percent derived from the mixed model analysis adjusted for baseline covariate.

[c]The TOST probability defined compatibility that a contrast was at least >SIE: 0.25–0.75, *possible* (*); 0.75–0.95, *likely* (**); 0.95–0.995, *very likely* (***); >0.995, *almost certain* (****). *Unclear* (without *) refers to outcomes where $p > 0.05$ for both increase and decrease relative to the SIE.

Another limitation related to the practicality of delivery of a dietary macronutrient supplement was that the background diet was uncontrolled. Participants completed a full 4-day diet recall diary at baseline and in the final week of intervention. The initial intention was to analyse diet for any change and if different consider as a covariate on the primary outcomes. Group mean total protein intake at baseline ($n = 24$) was 91 (SD 32) $g \cdot d^{-1}$ (~1.0 $g \cdot kg^{-1} \cdot d^{-1}$), and at week 10, CON 88 $g \cdot d^{-1}$ (34), WHEY 91 $g \cdot d^{-1}$ (14), with the post-pre difference between groups unclear (10%, 90% CI −14, 42). We concluded post hoc that sampling may not represent the dietary intake during the whole 10-week intervention making any inference unreliable.

In conclusion, in this exploratory trial, whey protein supplementation coupled with chronic exercise training increased insulin-stimulated skeletal muscle microvascular blood perfusion in men with T2DM, without clear additional effect on blood pressure, muscle angiogenic or mitochondrial density. The positive association between microvascular hemodynamic response and insulin-stimulated glucose disposal suggests that further investigation is warranted into the effects of dietary protein modification with exercise therapy on microvascular function in insulin resistant populations, but at this time insufficient evidence supports adding whey protein supplementation to the established effective benefits of intense exercise as a clinical therapy in T2DM.

Conflict of interest statement

The authors declare no conflicts of interest.

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