Acute cocoa flavanol supplementation improves muscle macro- and microvascular but not anabolic responses to amino acids in older men

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ABSTRACT

The anabolic effects of nutrition on skeletal muscle may depend on adequate skeletal muscle perfusion, which is impaired in older people. Cocoa flavanols have been shown to improve flow-mediated dilation, an established measure of endothelial function. However, their effect on muscle microvascular blood flow is currently unknown. Therefore, the objective of this study was to explore links between the consumption of cocoa flavanols, muscle microvascular blood flow and muscle protein synthesis (MPS) in response to nutrition in older men. To achieve this objective leg blood flow (LBF), muscle microvascular blood volume (MBV) and MPS were measured under postabsorptive and postprandial (I.V glamin, dextrose to sustain glucose ~7.5 $mmol \cdot l^{-1}$) conditions in 20 older men. Ten of these men were studied with no cocoa flavanol intervention and a further 10 were studied with the addition of 350 mg of cocoa flavanols at the same time as nutrition began. Leg [femoral artery] blood flow was measured by Doppler ultrasound, muscle MBV by contrast-enhanced ultrasound (CEUS) using Definity^{TM} perflutren contrast agent and MPS using [1, 2-$^{13}C_2$] leucine tracer techniques. Our results show that although older individuals do not show an increase in LBF or MBV in response to feeding, these absent responses are apparent when cocoa flavanols are given acutely with nutrition. However this restoration in vascular responsiveness is not associated with improved MPS responses to nutrition. We conclude that acute cocoa flavanol supplementation improves muscle macro- and microvascular responses to nutrition, independently of modifying muscle protein anabolism.

Key Words: Blood flow, Muscle protein synthesis, Cocoa, Aging, Metabolism

Word Count: 243

INTRODUCTION

Skeletal muscles facilitate whole-body locomotory function but also represent the body's largest metabolically active protein reservoir and glucose disposal site (Wolfe 2006). Given these crucial functional and metabolic duties ascribed to muscles, age-related sarcopenia (i.e., atrophy) represents a major health problem. Indeed, in older-aged people habitually suffer reductions in strength and power, which are exacerbated by sarcopenia and culminate in dynapenia and loss of independence, resulting in a cyclical syndrome of frailty (Fried et al. 2001).

In addition to frailty, the link between ageing, sarcopenia and the development of cardiovascular and metabolic-related diseases is striking. For example, decreased arterial compliance in older age leads to hypertension and coronary artery disease (DeSouza et al. 2002; Dinenno et al. 1999; Lind & Lithell 1993). In addition, when compared to younger individuals, older individuals exhibit 20-30% reductions in limb conduit artery blood flow under both postabsorptive (Donato et al. 2006) and postprandial conditions (Skilton et al. 2005), due to both a decrease in capillary number (Coggan et al. 1992) and increased sensitivity to vasoconstrictors such as alpha-adrenergic innervation (Barrett-O'Keefe et al. 2013). Such blunted blood flow responses may contribute to age-related declines in anabolic responses to feeding (Durham et al. 2010), by reducing delivery and/or utility of insulin and amino acids (AA) in muscle (Clark et al. 2003; Timmerman et al. 2010). Thus, it is likely that determining strategies for maintaining limb blood flow in older individuals will be important for both maintaining muscle mass and reducing cardiovascular/ metabolic disease risk.

In addition to total limb blood flow, another mediator of insulin and AA delivery to the muscle is the distribution of blood flow between so-called nutritive and non-nutritive routes (Clark et al. 2006; Clark 2008; Durham et al. 2010), as reduced nutritive blood flow may contribute to the anabolic resistance observed with ageing (Cuthbertson et al. 2005; Wilkes et al. 2009). We have previously shown that in young individuals pharmacological enhancement of 'nutritive' muscle microvascular blood flow was not associated with increases in muscle protein synthesis (Phillips et al. 2014). However, in older individuals it has been reported that there may be a positive relationship between enhanced muscle microvascular blood flow and muscle protein anabolic responses to nutrition (Timmerman et al. 2012).

A substantial body of epidemiological and experimental evidence has established that dietary interventions increasing the intake of plant-derived nutritional sources are inversely associated with cardiovascular disease risk (Joshipura et al. 2001). These beneficial effects have been linked to flavanols, a sub-group of flavonoids – the polyphenolic family of anti-oxidant chemicals, which are found in abundance in various foods (Heiss et al. 2005), including, but not limited to red wine, black tea, apples, onions and cocoa (Campia & Panza 2008). The benefits of flavanol consumption are purported to be due to increased nitric oxide (NO) synthase activity and therefore an increased supply of bio-active NO (Fisher et al. 2003; Karim et al. 2000), with numerous studies showing that both acute (Duffy et al. 2001; Engler et al. 2004; Heiss et al. 2003; Leikert et al. 2002) and chronic flavanol supplementation can improve endothelium dependent vasodilation (Fisher et al. 2003; Karim et al. 2000). Interest in flavanols spread when initial beliefs that the acidic environment of the stomach degraded flavanol oligomers were disproven (Kwik-Uribe & Bektash 2008; Rios et al. 2002). Indeed once in the upper intestine, flavanols monomers and dimers can be absorbed and cocoa

flavanols monomers/ dimers have been shown to appear in plasma within 30 to 60 minutes post-consumption (Richelle et al. 1999; Schroeter et al. 2006).

Interest in cocoa flavanols in particular was triggered by the finding that Kuna Indians living off the coast of Panama had a significantly lower incidence of vascular-related diseases compared to those living in Panama city (Bayard et al. 2007; Hollenberg et al. 1997), with dietary habits, and specifically the high intake of home-prepared cocoa found to be the most likely candidate for this disparity (Fisher & Hollenberg 2005). As with other forms of flavanols numerous studies have provided evidence that cocoa flavanols can enhance endothelial function in healthy individuals (Monahan et al. 2011), smokers (Heiss et al. 2005), diabetics (Balzer et al. 2008), hypertensive patients (Grassi et al. 2005), patients with coronary artery disease (Heiss et al. 2010) and heart transplant recipients (Flammer et al. 2007). However, whether cocoa flavanols can improve microvascular responses to nutrition is wholly unknown.

Our objective was to determine the effect of acute cocoa flavanol supplementation on muscle microvascular blood flow and subsequent muscle protein synthetic responses to nutrition using an I.V feeding strategy. We hypothesised that acute cocoa flavanol supplementation would improve muscle microvascular blood flow responses to feeding and that this would be associated with improved muscle protein synthetic responses.

MATERIALS AND METHODS

Subject characteristics

We recruited two groups of older men who were matched for age, body mass index (BMI) and baseline physiology (**Table 1**). The first group (Control (CON): n=10, 72.3±1.4 y; BMI

26.5±1.2 kg·m^2) were studied with no cocoa flavanol supplementation. The second group were studied with an acute dose of 350 mg cocoa flavanol supplementation (CocoaVia®, Mars Botanical, Mars Inc., MD, USA) during their study day (Cocoa (CC): n=10, 70.6±1.4 y; BMI 25.0±0.8 kg·m^2). The 350 mg dose was chosen as this dose is similar to the acute dose at which improvements in indices of cardiovascular function have been observed (Francis et al. 2006; Mogollon et al. 2013; Sansone et al. 2015) and closely represents the dose given during chronic intervention studies (Balzer et al. 2008; Engler et al. 2004; Heiss et al. 2015; Muniyappa et al. 2008).

All subjects were initially screened by means of a medical questionnaire, physical examination and resting ECG, with exclusions for overt muscle wasting (>2 SD below age norms) (Baumgartner et al. 1998), metabolic, respiratory or cardiovascular disorders or other signs and symptoms of ill-health. All subjects had normal blood chemistry, were normotensive (BP<140/90) and were not prescribed medication. All subjects performed activities of daily living and recreation but did not routinely participate in formal exercise regimes. All subjects gave their written, informed consent to participate after all procedures and risks were explained. This study was approved by The University of Nottingham Ethics Committee, complied with the Declaration of Helsinki and was registered at clinicaltrials.gov (NCT01734616).

Acute studies

Subjects were instructed to refrain from strenuous activity for 72 h prior to each study day and from alcohol and caffeine for 24 h. Subjects fasted from 2100h the night before (water *ad libitum*) and arrived to the laboratory via non-active means at 0900h. Subjects then had polyethylene catheters inserted into the antecubital veins of both arms (one for tracer infusion and the other for perflutren microbubble infusion) and the femoral vein of one leg (for venous

blood sampling). Blood samples were taken every 20 min. A primed, continuous infusion (0.7 $mg \cdot kg^{-1}$, 1 $mg \cdot kg \cdot h^{-1}$) of [1, 2-$^{13}C_2$] leucine tracer (99 atoms %; Cambridge Isotopes Ltd, Cambridge, MA) was started at 0 min and maintained for the duration of the study with an increase to 1.2 $mg \cdot kg \cdot h^{-1}$ at 130 min when a continuous I.V infusion of Glamin was started (102$mg \cdot kg \cdot h^{-1}$, providing ~20 g AA) and 20% dextrose infused at a rate to maintain blood glucose between 7-7.5 $mmol \cdot l^{-1}$ (for Glamin composition see Table 2). The CC group was also provided with a single dose of 350 mg cocoa flavanol supplementation as I.V nutrition began. Muscle biopsies of *m. vastus lateralis* were taken at 0, 120 and 250 min using the conchotome biopsy technique (Dietrichson et al. 1987), with 1% lignocaine as local anaesthetic. The muscle tissue was washed in ice-cold saline to remove excess blood with visible fat and connective tissue removed, before being snap frozen in liquid nitrogen and stored at -80°C until further analysis (**Figure 1**).

Leg Blood Flow (LBF)

After 50 min lying supine, measurements of LBF (femoral artery) were made over 40 min, with these values were designated as BASAL. This was repeated at 180 min (50 min after the start of Glamin and dextrose infusions) with these measurements labelled as FED. A mean value from three measurements on each leg was used to obtain the final value, no significant differences were observed between the three measurements. LBF was measured using Doppler ultrasound (iU22; Philips Ultrasound, CA, USA). All measurements were taken with the subject supine, with no visual or aural stimuli. A linear array 9-3 MHz frequency probe (Philips Ultrasound) was used to measure mean blood velocity and arterial lumen diameter in the common femoral artery of one leg. Measurements were made 2-3 cm proximal to the bifurcation of the femoral artery to minimize the effect of turbulence; the insonation angle was <60°. Arterial lumen diameter (mm) was measured by video callipers for each

measurement and defined as the maximum distance between the media-adventitia interface of the near wall and the lumen-intima interface of the far wall of the vessel. LBF ($l.min^{-1}$) was calculated as: mean blood velocity ($cm.sec^{-1}$) × π × (femoral artery radius (mm))^2)/1000 × 60.

Microvascular Blood Volume (MBV)

At 110 min (BASAL) and 240 min (110 min after the start of nutrition (FED)), measurements of MBV in the *m. vastus lateralis* were made using contrast enhanced ultrasound (CEUS), as previously described (Sjøberg et al. 2011). In brief, a linear array transducer (L9-3 MHz; Philips Ultrasound) was fixed to the thigh for the duration of the study to allow cross-sectional imaging of the *m. vastus lateralis*. A 1.5ml suspension of perflutren (Definity; Lantheus Medical Imaging, MA, USA) microbubbles was diluted to 20ml and infused at a rate of $1.2ml \cdot min^{-1}$. Real-time imaging was performed at a low-mechanical index (0.08) for 9 min to allow attainment of a steady-state microbubble signal. After this period a high mechanical index (1.20) flash was used to destroy the microbubbles, allowing recording of the replenishment of the microbubbles in the vasculature within the ultrasound beam during a 45 sec replenishment period. The acoustic index (AI) generated from the resonating microbubbles is proportional to the microbubble concentration in the region of interest (ROI) (Wei et al. 1998). Four destruction-replenishment cycles were recorded for each MBV measurement (BASAL and FED).

Data from these recordings were exported to quantification software (Q-Lab; Philips Ultrasound) for analysis. ROI were drawn free-hand and selected to be free of connective tissue and large vessels. The selected ROI was copied for each recording to ensure that the ROI was identical for both BASAL and FED conditions. The AI obtained during the first 0.5 sec was averaged and subtracted from the AI recorded during the remaining time to eliminate

background noise and contribution from any rapid filling vessels in the ROI. Calculations were made in accordance to Wei *et al.,* with AI vs. time curves fitted to the equation: $y = A[1 - e^{-\beta(t-\beta_t)}]$, where t is time (s), B_t the time used for background subtraction, y is the acoustic intensity at any given t, A is the plateau AI defined as MBV and β is the flow rate constant (1/s) that determines the rate of rise of AI, proportional to microvascular flow velocity (MFV; (Krix et al. 2005; Wei et al. 1998)). The product of Aβ with the unit volume $blood^{-1} \cdot$volume $tissue^{-1} \cdot second^{-1}$ is proportional to microvascular blood flow (MBF) (Mitchell et al. 2013).

Muscle Protein Synthesis (MPS)

Approximately 20mg of muscle tissue was used for measures of MPS, as previously described (Kumar et al. 2012). In brief, the myofibrillar pellet was precipitated by homogenisation and centrifugation with the soluble myofibrillar protein then precipitated by perchloric acid. Protein-bound AA's were released by acid hydrolysis in Dowex H^+ resin slurry before being purified by ion exchange chromatography on Dowex H^+ resin. The AA's were then derivatized as their n-acetyl-N-propyl esters as previously described (Wilkinson et al. 2008). Incorporation of [1, 2-$^{13}C_2$] leucine into protein was determined by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XP, Thermofisher Scientific, Hemel Hempstead, UK) using our standard techniques (Babraj et al. 2005), with the fractional synthetic rate (FSR) of myofibrillar protein determined from the incorporation of [1, 2-$^{13}C_2$], using the precursor labelling of venous α-KIC between subsequent muscle biopsies (Kumar et al. 2009). Plasma phenylalanine concentrations were measured via our standard techniques using a $^2H_2$ phenylalanine internal standard, with reference to a standard curve of known concentration (Phillips et al. 2015).

Immunoblotting and Plasma Insulin

To investigate the possible effects of cocoa flavanols via enhanced LBF/ MBV on anabolic signalling we measured protein phosphorylation of AKT and P70S6K1 (as likely indicators of such activity) in response to feeding. We also measured protein phosphorylation of endothelial nitric oxide synthase (eNOS) to explore possible mechanisms for the increased responses in LBF/ MBV we observed with cocoa flavanol supplementation. The supernatant (sarcoplasmic fraction) obtained from the myofibrillar preparation described above, was standardized to a protein concentration of 1 $mg \cdot ml^{-1}$ by dilution with Laemmli buffer, mixed and heated at 95°C for 5 min before 15 μg of protein/lane was loaded on to Criterion XT Bis-Tris 12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for 60 min. Proteins were electroblotted on to 0.2 μm PVDF membranes (Bio-Rad) at 100 V for 30 min and membranes blocked in 5% low-fat milk in TBS-T (Tris Buffered Saline and 0.1% Tween-20; both Sigma-Aldrich, Poole, UK) for 1 h; membranes were rotated overnight with primary antibody (Cell Signalling Technology, Boston, USA) at 1:2000 at 4°C. Membranes were washed (3×5 min) with TBS-T and incubated for 60 min at room temperature with HRP-conjugated anti-rabbit secondary antibody (Cell Signalling Technology, Hertfordshire, UK), before further washing (3×5 min) with TBS-T and incubation for 5 min with ECL reagents (Enhanced chemiluminescence kit; Immunstar; Bio-Rad, Hemel Hempstead, UK). Blots were imaged and quantified by peak density within the linear range using the Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK). Coomassie staining was used to correct for loading (Welinder & Ekblad 2011).

Postabsorptive and fed plasma insulin concentrations were measured using undiluted samples and high-sensitivity insulin enzyme linked immunosorbent assays (ELISA; DRG Instruments GmbH, Marburg, Germany) according to manufacturer's protocol.

Statistical analyses

Statistical analyses were performed using Graph Pad Prism Version 6.00 (La Jolla, CA, USA). All data are reported as means ± SEM with the threshold for significance set at $P<0.05$ with trends reported as $P<0.1$. We applied Students t-tests to assess LBF, MBV, MPS, plasma insulin and α-KIC responses to nutrition and ANOVA with Bonferroni post-hoc analysis to compare between the groups.

RESULTS

Measures of LBF

The CON group did not demonstrate increased LBF in response to nutrition (0.32±0.04 vs. 0.32±0.04 $l \cdot min^{-1}$), a response that was apparent in the CC group (0.47±0.03 vs. 0.54±0.04 $l \cdot min^{-1}$, $P<0.01$). Although this is likely partly attributable to the higher BASAL LBF in the CC group (0.47±0.03 vs. 0.32±0.04 $l \cdot min^{-1}$, $P<0.05$), this disparate response to nutrition, resulted in FED LBF values that were significantly higher in the CC group compared to the CON group (0.54±0.04 vs. 0.32±0.04 $l \cdot min^{-1}$, $P<0.05$) (**Figure 2**).

Measures of MBV

In agreement with our previous work and the work of Sjoberg *et al.,* we found that with an infusion of 1.2$ml \cdot min^{-1}$ of Definity microbubbles a plateau signal was achieved at approximately 6 min (Phillips et al. 2014; Sjøberg et al. 2011). Based on this an infusion time of 9 min was used before the measurements of MBV were obtained. The combined I.V nutrition strategy of 20% Dextrose at a rate to achieve blood glucose of 7-7.5$mmol \cdot l^{-1}$ and 102$mg \cdot kg \cdot h^{-1}$ of Glamin (following a 34$mg \cdot kg^{-1}$ prime) was not able to elicit a significant increase in MBV in the CON group (AI: 1.52±0.18 vs. 1.62±0.19; 6%). The CC group did demonstrate a significant (20%) increase in MBV in response to nutrition (1.24±0.17 vs.

1.43±0.15, P<0.01; **Figure 3**). Neither group demonstrated a significant increase in flow rate constant (β-value) in response to nutrition (CON: BASAL, 0.13±0.02 vs. FED, 0.13±0.02; CC: BASAL, 0.11±0.01 vs. FED, 0.12±0.01). In keeping with the results for MBV, MBF (MBV×β-value) increased only in the CC group in response to nutrition (CON: BASAL, 0.21±0.05 vs. FED, 0.21±0.04; CC: BASAL, 0.15±0.03 vs. FED, 0.17±0.03, P<0.05).

Plasma Amino Acid and Keto-acid Concentrations

Postabsorptive plasma phenylalanine concentrations were similar between the groups (CON: 67.49 ± 3.34 vs. CC: 66.41 ± 2.08 μM) and increased significantly, but to the same extent in response to feeding in both groups (CON: 115.94 ± 4.34 vs. CC: 120 ± 4.13 μM; P<0.001). Steady-state KIC enrichment was also observed throughout both conditions (BASAL, CON: 4.81 ± 0.21 vs. CC: 5.33 ± 0.21; FED, CON: 5.79 ± 0.21 vs. CC: 6.38 ± 0.18 APE) and was not significantly different between the groups.

Myofibrillar MPS

Contrary to the results for LBF and MBV, both groups demonstrated significant increases in MPS in response to feeding (CON: BASAL, 0.052±0.006 vs. FED, 0.074±0.006%·h^{-1}; CC: BASAL, 0.042±0.005 vs. FED, 0.068±0.010%·h^{-1}, both P<0.01). Neither the magnitude of this response (CON: 57±16 vs. CC: 73±20%), nor the BASAL (CON: 0.052±0.006 vs. CC: 0.042±0.005%·h^{-1}) or FED (CON: 0.074±0.006 vs. CC: 0.068±0.010%·h^{-1}) MPS values were significantly different between the groups (**Figure 4**).

Plasma Glucose and Insulin

Despite the greater increase in LBF and MBV in response to feeding in the CC group this was not associated with an increased dextrose infusion to maintain blood glucose at 7-7.5 mmol·l^{-1}, as there was no significant difference between the groups (AUC, CON:

560.40±41.06 vs. CC: 479.80±42.75 ml·min^{-1}; Figure 5). This was also true for FED plasma insulin values, which were not significantly different between the groups (AUC, CON: 30.72±9.15 vs. CC: 53.02±9.51 μUnits·ml^{-1}; Figure 5).

Immunoblotting

There were no differences in BASAL or FED phosphorylation values of P70S6K1 (CON: BASAL, 1.01±0.03; FED, 1.05±0.04; CC: BASAL, 0.91±0.05; FED, 0.81±0.09 Arbitrary unit of optical density (AU)), AKT (CON: BASAL, 1.18±0.06; FED, 1.16±0.16; CC: BASAL, 1.75±0.11; FED, 1.52±0.16 AU) or eNOS (CON: BASAL, 0.90±0.03; FED, 1.00±0.16; CC: BASAL, 1.02±0.06; FED, 1.17±0.14 AU) between the groups. Neither group demonstrated a significant increase in phosphorylation of any of the aforementioned targets in response to nutrition.

DISCUSSION

In this study, we have for the first time explored the effect of acute cocoa flavanol supplementation upon muscle vascular and muscle protein anabolic responses to nutrition in older individuals. We report that LBF and MBF responses to feeding do not appear to be rate-limiting for MPS in older men since acute cocoa flavanol supplementation alongside mixed macro-nutrient provision improved macro- and microvascular responses to nutrition but this did not enhance anabolic responses to nutrition.

Previously we have shown that resistance exercise training (RE-T) can restore age-related reductions in LBF in response to feeding (Phillips et al. 2012), and more recently have discovered the same to be true for MBF (Phillips et al. 2015). This intervention however, required 20 weeks of whole-body RE-T, a commitment that even irrespective of time is not

achievable by all older individuals due to physical and/ or socio-economic constraints. In an attempt to find a more time-efficient method to improve vascular responses to nutrition, we employed a strategy whereby we infused methacholine - a pharmacological vasodilator, into the femoral artery of young, healthy males to assess the impact of pharmacological increases in LBF on both MBF and MPS. Interestingly in these individuals we found that methacholine, as expected, was able to increase macro- and microvascular responses to mixed macronutrient (I.V) "feeding", but that this was not associated with improved MPS responses (Phillips et al. 2014). We rationalised that this lack of association may be due to already optimal vascular and muscle protein synthetic responses to nutrition in the young that are not apparent in older individuals (Cuthbertson et al. 2005; Phillips et al. 2012). In addition, we considered the difficulties in applicability of a pharmacological agent as a health-based intervention tool and instead moved towards using cocoa flavanols, a neutraceutical agent that has already been shown to be well tolerated (Balzer et al. 2008) and effective at improving vascular function in a wide variety of healthy and disease-based cohorts (Heiss et al. 2010).

In young and middle aged subjects, a well-defined response to nutrient intake is increased limb arterial blood flow (Raitakari et al. 2000) but as we reported previously, such responses are reduced or absent in older people (Phillips et al. 2012). Here we have again shown that age-related decrements in LBF are associated with impairments in muscle MBV responses to feeding, findings that are consistent with reports of decrements in fed-state forearm MBF (inferred from indirect measurements) (Skilton et al. 2005) and also the absence of increased muscle MBF in older people fed EAA and sucrose (Timmerman et al. 2012). Whereas it is well established that acute bouts of exercise enhance microvascular perfusion in both human (Vincent et al. 2006) and rodent muscle (Sjøberg et al. 2011), the effects of an acute

neutraceutical intervention (in this case cocoa flavanols) on muscle MBF in response to nutrition, have until now remained poorly defined. Here, we have shown that 350 mg cocoa flavanols improved nutrient-dependent increases in both LBF and muscle MBF in older men.

Contrary to the purported mechanisms for exercise-training induced improvements in MBF (which include angiogenesis (Gavin et al. 2007), capillarization (McCall et al. 1996) and vascular remodelling of the arteriolar network (Weber et al. 2013)), independent mechanisms must underlie the results observed in this acute intervention study as the compressed time-frame negates the possibility for the mechanisms outlined above. Based on the well-established improvements in endothelium dependent vasodilation afforded by the consumption of cocoa flavanols, possible mechanisms for this include altered: metabolite profiles (Schroeter et al. 2006), cell signaling pathways, gene expression and/ or protein activity (Middleton et al. 2000; Scalbert et al. 2005), although our results suggest that this improvement is not linked to insulin signaling pathways as there were no differences in plasma insulin, proximal insulin signaling (AKT) or dextrose requirement to maintain a fixed blood glucose between the groups.

Although MPS increases after oral consumption (Atherton et al. 2010) or I.V infusions of essential AA are well accepted (Anthony et al. 2000; Bennet et al. 1990; Millward 2012) previous work by our group (Cuthbertson et al. 2005) and others (Volpi et al. 2000) has demonstrated ‘anabolic resistance’, whereby older men demonstrate blunted (compared to those in younger men) fed-state increases in MPS. Such anabolic resistance has not only been conjectured by us and others to contribute to the sarcopenia of ageing but it also appears to be a common feature of many kinds of muscle atrophy (e.g. with immobilization (Glover et al. 2008), reduced physical activity (Breen et al. 2013), heart failure (Toth et al. 2010), obesity

(Guillet et al. 2009; Murton et al. 2015) and cancer (Williams et al. 2012)), suggesting a final common pathway for a variety of insults resulting in diminished anabolism. Whereas the regulatory mechanisms of anabolic resistance remain poorly defined, possible links between reduced muscle MBF and diminished anabolic responses to plasma nutrient availability may be a general feature of a number of conditions.

We have now defined the relationship between fed-state muscle protein anabolism and MBF in older individuals and against our expectations; we found that despite cocoa flavanols resulting in greater (in comparison with an independent age-matched group) fed-state LBF and MBF responses, increases in MPS were similar between groups. This finding was based on a 2-hour window of fed-state MPS investigation, the postprandial period that has been well defined by our group (Atherton et al. 2010; Mitchell et al. 2015a; Mitchell et al. 2015b). Indeed, our recent work has demonstrated that in older individuals, in response to both 20 g whey protein and 3 g leucine-enriched essential amino acids, anabolic responses are apparent over the 0-2 hour period but not evident over the subsequent 2-4 hours. Therefore it would seem that interventions aimed at increasing the acute amplitude of MPS are most critical and that the duration appears to be more fixed (in the absence of accompanying exercise (Drummond et al. 2008). Although using this study design we cannot be certain that longer measures of MPS would not have shown increases in MPS in the CC group, we predict this would not be the case.

We also found that although cocoa flavanols improved vascular responses to nutrition, this was not associated with any enhancement of eNOS signaling. Indeed, the increases in MPS in both groups in response to feeding were not associated with enhancement of P70S6K1 or AKT signaling. These lack of associations are not however surprising given that the

relationship between the degree of fed-state AKT phosphorylation and that of its supposed metabolic effects are poorly related (Greenhaff et al. 2008).

We acknowledge some potential study limitations. We were unable to obtain data on flavanol bioavailability for this study. However, flavanol plasma availability is known to occur within 30-60 min of consumption (Richelle et al. 1999), demonstrating that flavanol bioavailability upon oral consumption occurs within our period of measurement and fits with our observation of an acute effect on blood flow. Due to the invasive nature of the acute studies we performed with multiple muscle biopsies and central (femoral venous) cannulation, we chose not to study the same cohort twice, choosing rather to minimize the sampling burden on our subjects. A fundamental limitation of the CEUS technique for measuring MBF is that it cannot be used to compare absolute values between individuals due to differences in the architecture of muscle blood vessels and therefore definition of ROI in different people; thus, we were unable to directly compare values between our subjects. However, delta changes within subjects in each group in response to a stimulus is a robust measure and thus the 20% feeding-induced increase in MBV in the CC group is comparable to the CON group that showed no significant change in MBV in response to our feeding regime. Furthermore, the effects (if any) of blood flow in modulating muscle protein turnover may depend, in a currently unknown way upon the mode of feeding and the composition or volume of the feed, so that our results may not be applicable to the effects of other patterns of feeding. For example, these results may not be fully representative of oral feeding, where additional factors such as gut hormones (e.g. incretins) may elicit an effect. However, although most of the non-essential AA's would be sequestered on first pass splanchnic extraction, the essential AA's (which impact MPS) would be largely peripherally available to muscle minimising the potential for route of delivery to effect our findings (Abdulla et al. 2014). Finally, we

acknowledge that these findings may not represent those found in an alternative cohort (e.g. those with co-morbidities or difference baseline characteristics). For example, it may be that in sarcopenic or obese individuals reduced MBF is indeed a facet of blunted responses to anabolic stimuli for which cocoa flavanols may be beneficial. Similarly this may also be true for older females who have been shown to have distinct anabolic responses to males (Smith et al. 2008, Smith et al. 2012) despite similarly blunted leg blood flow responses to feeding (Bukhari et al. 2015).

To conclude, we have shown that macro- and microvascular blood flow responses to mixed nutrition (I.V AA (~20 g AA, 8 g EAA (equivalent to a small chicken portion or 20 g whey protein) and dextrose) can be improved by acute cocoa supplementation in older men, but that this improvement does not enhance muscle protein anabolic responses to nutrition. These data imply that neutraceutical strategies based on cocoa flavanol supplementation could be used to improve muscle microvascular recruitment in postprandial periods, which although may not have positive effects on muscle protein metabolism, could positively impact on other aspects of metabolism (i.e. insulin sensitivity, glucose clearance) in older individuals. We believe that this, and the potential of long-term cocoa flavanol supplementation to improve different elements of muscle metabolism warrant further investigation.

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Conflict of interests

No authors have any conflicts of interest to declare.

Authors' contributions to manuscript

B.E.P., J.P.W. and K.S designed the research; B.E.P., K.V., M.C.L., J.P.W. and K.S conducted the research; B.E.P., P.J.A., M.C.L. and K.S analyzed the data; B.E.P., P.J.A. and K.S wrote the paper; B.E.P. and K.S had primary responsibility for final content of the paper and all authors read and approved the final manuscript.

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TABLES

Table 1. Subject characteristics

	Control	Cocoa
Body mass Index ($kg \cdot m^2$)	26.5±1.2	25.0±0.8
Lean whole body mass (kg)	52.1±1.66	53.4±1.22
Lean leg mass (kg)	8.42±0.43	8.71±0.29
Fasting glucose ($mmol \cdot l^{-1}$)	5.1±0.1	5.3±0.2
Systolic BP (mmHg)	132±4	134±5
Diastolic BP (mmHg)	85±3	83±3
Resting HR (bpm)	66±2	60±3

Table 2. Glamin® composition per 1000 mL

Constituent	Content (g)	Constituent	Content (g)
Alanine	16.0	Leucine	7.90
Arginine	11.30	Lysine-Acetate:	12.70
Aspartic Acid	3.40	*Corresp to Lysine*	9.0
Glutamic Acid	5.60	Methionine	5.60
Glycyl-Glutamine:	30.27	Phenylalanine	5.85
Corresp to Glycine	10.27	Proline	6.80
Corresp to Glutamine	20.0	Serine	4.50
Glycyl-Tyrosine:	3.45	Threonine	5.60
Corresp. to Glycine	0.94	Tryptophan	1.90
Corresp. to Tyrosine	2.28	Valine	7.30
Histidine	6.80	Citric Acid	to pH 5.8
Isoleucine	5.60	Water	to 1000 mL

FIGURE CAPTIONS

Figure 1. Acute study protocol

Figure 2. Femoral artery blood flow in old male subjects in BASAL (post-absorptive) and FED conditions (102mg·kg·h^{-1} Glamin and 20% dextrose to maintain blood glucose between 7-7.5 mmol·l^{-1}) with (Cocoa) and without (Control) the addition of 350 mg cocoa flavanols. Values are means ± SEM for n=10 in each group. **P<0.01 vs. BASAL in the same group; ^ P<0.05 vs. Control in the same condition. BASAL to FED comparisons via paired student's t-test. Group comparisons via ANOVA with Bonferroni post-hoc analysis.

Figure 3. Microvascular refilling curves after destruction of Definity microbubbles in old male subjects in BASAL (post-absorptive) and FED conditions with (Cocoa; C) and without (Control; A) 350 mg cocoa flavanols. Microvascular blood volume presented as the plateau value acoustic index (A value) in old male subjects in BASAL and FED conditions with (Cocoa; D) and without (Control; B) 350 mg cocoa flavanols. Values are means ± SEM for n=10 in each group. **P<0.001 vs. BASAL. Analysis via paired student's t-test.

Figure 4. Muscle protein synthesis in response to feeding with (Cocoa) and without (Control) 350 mg cocoa flavanols in old male subjects. Values are means ± SEM for n=10 in each group. **P<0.01 vs. BASAL. BASAL to FED comparisons via paired student's t-test. Group comparisons via ANOVA with Bonferroni post-hoc analysis.

Figure 5. Dextrose infusion (20%) required to maintain a blood glucose value of 7-7.5 mmol·l^{-1} and plasma insulin values in response to feeding with (Cocoa) and without (Control)

350 mg cocoa flavanols in old male subjects. Values are means ± SEM for n=10 in each group.

FIGURES

Figure 1

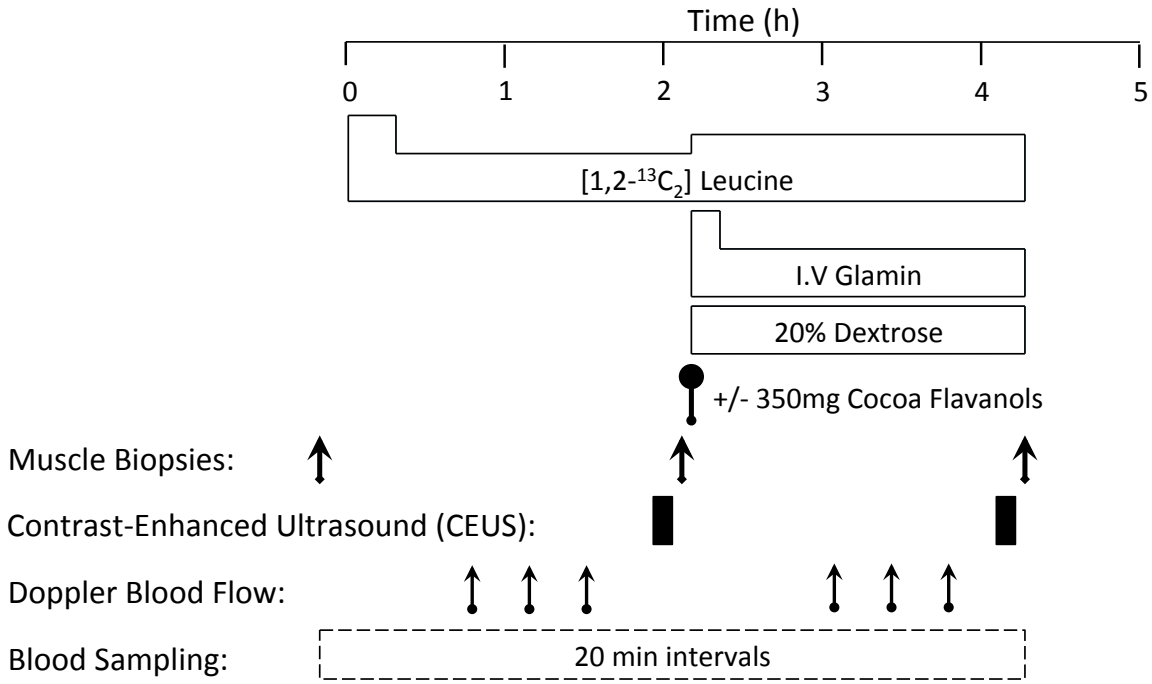


Figure 2

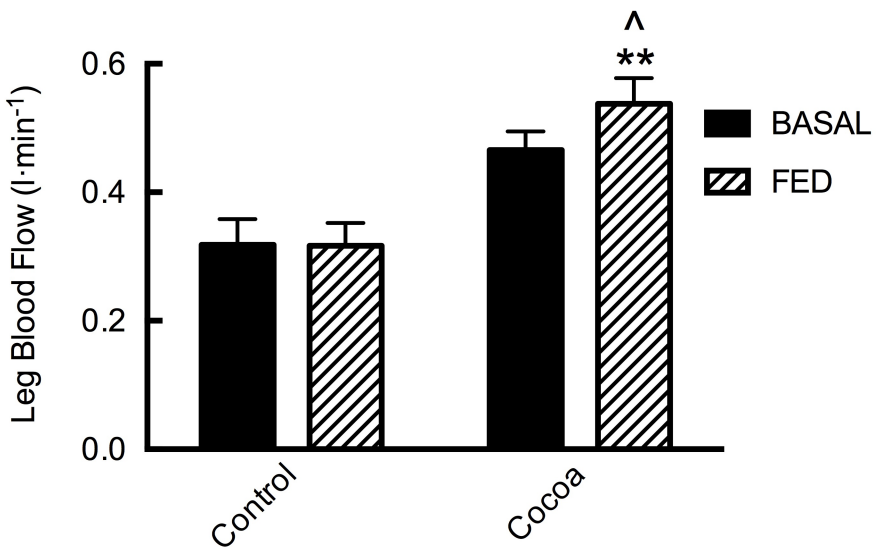


Figure 3

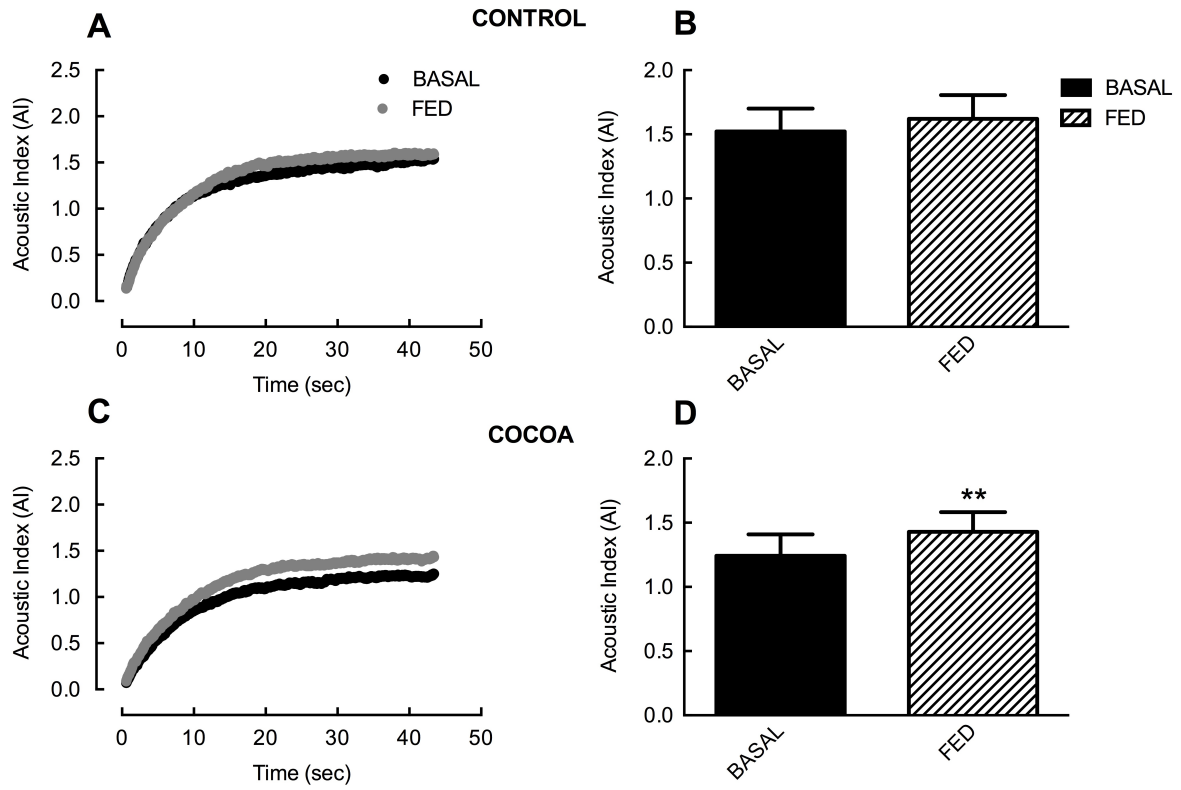

A
CONTROL
BASAL
FED
Acoustic Index (AI)
Time (sec)
B
BASAL
FED
C
COCOA
D
**

Figure 4

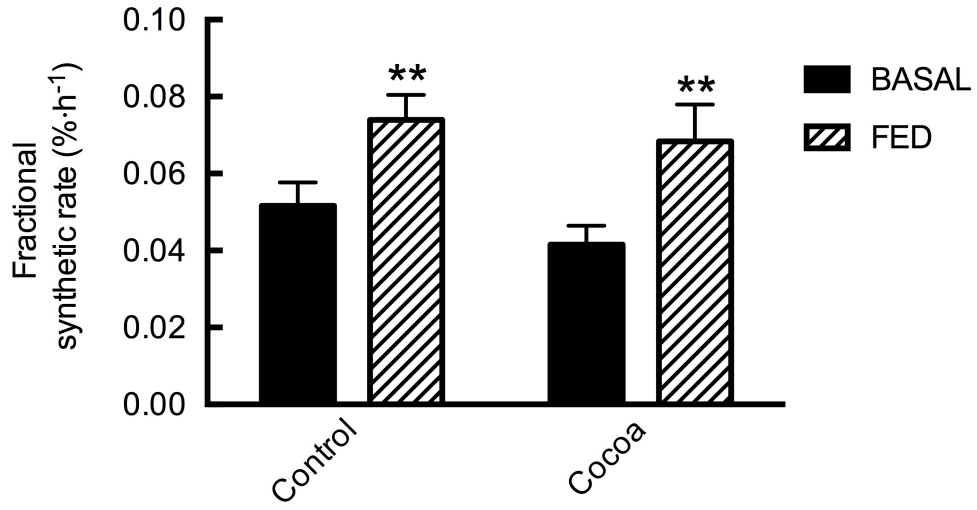
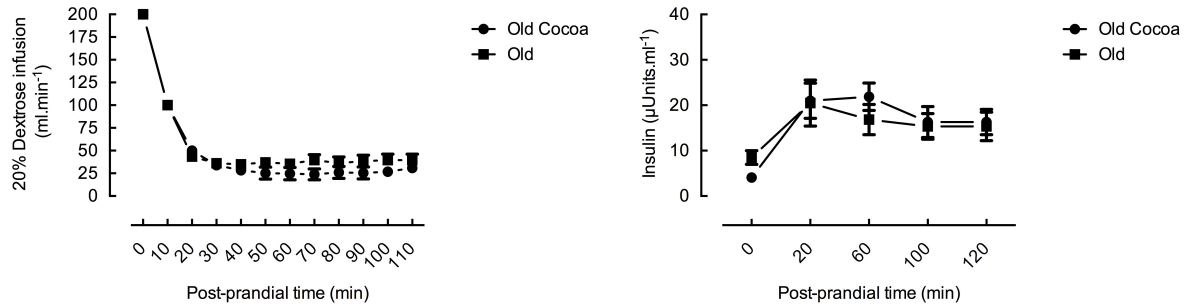

0.10
0.08
0.06
0.04
0.02
0.00
Fractional synthetic rate (%·h-1)
**
**
BASAL
FED
Control
Cocoa

Figure 5


20% Dextrose infusion (ml.min-1)
200
175
150
125
100
75
50
25
0
0 10 20 30 40 50 60 70 80 90 100 110
Post-prandial time (min)
Old Cocoa
Old
Insulin (µUnits.ml-1)
40
30
20
10
0
0 20 60 100 120
Post-prandial time (min)
Old Cocoa
Old