Impact of methionine restriction on muscle aerobic metabolism and hypertrophy in young and old mice on an obesogenic diet

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ABSTRACT

Methionine restriction (MR) reduces inflammation and increases longevity. We studied the effects of MR (0.17% kCal methionine, 10% kCal fat) and MRþhigh-fat diet (HFD) (0.17% methionine, 45% kCal fat) and overload-induced hypertrophy on inflammation, angiogenesis and mitochondrial activity in the hind-limb muscle in 10- and 26-month-old male C57BL/6J mice. Plasma IL-6 concentrations were higher in old compared to young mice. M. plantaris hypertrophy was accompanied by increased p-Akt, without a significant change in Akt and VEGF levels. In young mice on a HFD or MRþHFD diet the SDH activity was higher than in those from mice on other diets, irrespective of overload. There were no significant differences in total NAD concentration in the m. gastrocnemius. MR enhanced the skeletal muscle hypertrophic response in old age that was accompanied with an increase in p-Akt without significant changes in muscle oxidative capacity, low-grade systemic inflammation, NAD, VEGF or Akt levels.

KEYWORDS

Methionine restriction; high-fat diet; ageing; inflammation; hypertrophy

Introduction

The global population of people older than 65 years is growing steadily (WHO 2018b). Ageing is a natural and unavoidable process associated with insulin resistance and sarcopenia, the age-related loss of muscle mass and function (Rosenberg 1989) that will ultimately result in the loss of independence. It is therefore, that there is an increasing interest to perhaps not reverse, but at least delay the ageing process.

In addition to ageing, in the Western World also obesity is rising to epidemic proportions (WHO 2018a). Obesity is defined as having a body mass index >30 kg-m-2 that is largely attributable to an accumulation of body fat. The visceral fat mass (VFM) is a major source of inflammatory cytokines (Pedersen 2009) and an increase in the VFM, as seen in obesity, is thus likely to contribute to the often- observed chronic low-grade systemic inflammation in older people. It has been shown that if the levels of the circulating inflammatory cytokines IL-6 and TNFa exceed a certain threshold, these are (a) associated with muscle weakness (Visser et al. 2002; Bian et al. 2017; Schaap et al. 2006) and (b) downregulation of IGF-1 resulting in impaired muscular strength (Barbieri et al. 2003), contributing to anabolic resistance (Rennie 2009). The age-related increase in IL-6 (Alberro et al. 2021; Hager et al. 1994) may thus be aggravated by obesity and contribute to the accelerated loss of muscle strength (Degens, Swaminathan, and Tallis 2021; Tallis et al. 2021).

Although resistance exercise is a well-established countermeasure against age-related loss of muscle mass and function (Peterson, Sen, and Gordon 2011; Harridge, Kryger, and Stensgaard 1999), the hyper- trophic response may be blunted in old age in both humans and rodents, perhaps due to the chronic low- grade systemic inflammation in old age (Degens 2010). Interestingly, methionine restriction (MR), a mimetic of caloric restriction (CR), has been shown to not only lower the low-grade systemic inflammation and increase longevity (Orgeron et al. 2014; Wanders et al. 2018), but also to enhance the over- load-induced hypertrophy of the m. plantaris in old mice (Swaminathan et al. 2021). As part of the blunted hypertrophy in old mice was due to impaired angiogenesis (Hendrickse et al. 2021; Ballak et al. 2016), the MR-induced improved hypertrophic response of old mice (Swaminathan et al. 2021) may well be attributable to enhanced angiogenesis.

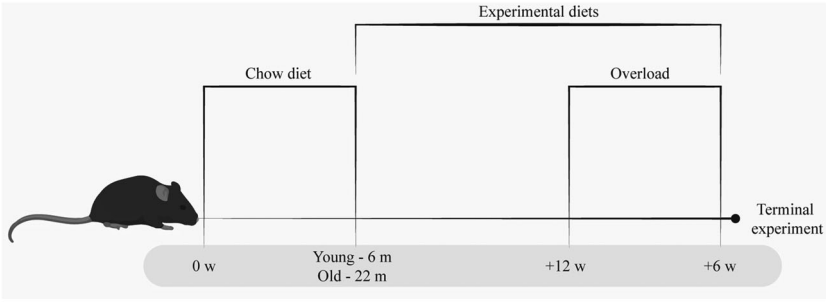


Figure 1. Timeline of the study with indication of the start and duration of chow diet and experimental diets, and the initiation and duration of overload of the right m. plantaris. Horizontal line shows the corresponding age of the animals/time elapsed. m: months; w: weeks.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and muscle fibre-specific VEGF knock-out mice exhibit less compensatory hyper- trophy (Huey et al. 2016) while on the other hand in old mice on sulphur amino acid restriction enhanced angiogenesis realised by an increased expression of VEGF (Longchamp et al. 2018). Likewise, angiogenesis induced by supplementation with nicotinamide mononucleotide, a booster of nicotinamide adenine dinucleotide (NAD), in old mice required the presence of VEGF (Das et al. 2018). Finally, Akt and p- Akt play are important mediators of hypertrophy (Bodine et al. 2001) and it remains to be seen whether the enhanced hypertrophic response during MR in old mice (Swaminathan et al. 2021) is associated with elevated muscle NAD and VEGF levels and enhanced Akt and p-Akt expression.

Mitochondria play a vital role in cellular ATP pro- duction, necessary for muscle contraction and viability of muscle cells. The mitochondrial dysfunction during ageing may impinge on proteostasis and result in a loss of muscle mass and function (Bellanti, Lo Buglio, and Vendemiale 2021), and may occur as a result of increased reactive oxidative species (ROS) production and lowered antioxidant defences (Miquel et al. 1980; McCormick and Vasilaki 2018). The low- grade systemic inflammation induced by obesity and old age, combined with deteriorated mitochondrial function, is especially relevant to older obese adults who have higher levels of muscular fat infiltration (Choi et al. 2016). Calorie restriction and exercise have been shown to delay the ageing- and obesity- associated impairment of mitochondrial function (Bhatti, Bhatti, and Reddy 2017; Ruetenik and Barrientos 2015). Similarly, it has been observed that while there was an absence of mitochondrial biogenesis, there was an increase in mitochondrial oxidative capacity in skeletal muscle of young-adult rats fed a MR diet (Perrone et al. 2010). Whether this increase in mitochondrial capacity in skeletal muscle after MR extends to old age and obesity is yet to be elucidated.

Therefore, the aim of our study was to assess whether the enhanced skeletal muscle hypertrophic response in old mice on MR is associated with elevated muscle levels of NAD and VEGF. In addition, we explored whether MR reduced circulating IL-6 and improved oxidative capacity in hypertrophied muscles of old mice on a high-fat diet. We hypothesised that MR lowers serum IL-6 and that the enhanced overload-induced hypertrophy in MR-fed old mice is associated with a higher muscle expression of p-Akt, VEGF and NAD.

Methods

Animals—diet and hypertrophy

All experiments were approved by the ethics commit- tee of the Lithuanian Republic Alimentary and Veterinary Public Office (No. G2-90 in 2018) and carried out in accordance with their guidelines and regulations. Figure 1 depicts the timeline of interventions. Male C57BL/6J mice had free access to water and standard chow (carbohydrate—66% kCal, protein— 21% kCal, fat—6% kcal, methionine—0.65% kCal) until the age of 6 (young-adult mice, n ¼ 38) or 22 months (old mice, n ¼ 32). The age of 22 months was chosen for the old group as that is the age of onset of an age-related decline in muscle mass in mice (Graber et al. 2015). Then they were sub-divided into the following groups: control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. Diets were purchased from Research Diets Inc. (New Brunswick, NJ, USA) and the diet compositions are shown in Table 1.

During the twelfth week of the dietary intervention, compensatory hypertrophy of the right plantaris muscles was induced in all mice by cutting the branches of the n. Ischiadicus supplying the

Table 1. Composition of chow and experimental diets (along with the corresponding Research Diets ID) fed to mice for the duration of the study.

Diet Composition	Chow	Control (A06071322)	MR (A18121002)	HFD (A06071309)	MR þ HFD (A18121001)
%Carbohydrate	66	72	72	36	37
%Protein	21	18	17	18	17
%Fat	6	10	10	46	46
%Methionine	0.65	0.49	0.17	0.61	0.17

MR: Methionine restricted diet; HFD: high fat diet (Adapted from Swaminathan et al. 2021). Nutrients are expressed as percentages of total calories (kCal).

m. gastrocnemius and *m. soleus* as close to their point of entry to the belly of the muscle as possible. A segment of each branch was removed to prevent reinnervation. The left plantaris muscle served as the internal control. The surgery was performed under anaesthesia (isoflurane—4% and O_2 at 2 L-min^{-1} until the animal did not respond to foot-pad-pinch, and then maintained with 1.5% isoflurane and 1 L-min^{-1} O_2).

Six weeks after overload surgery, the animals were euthanised with an overdose of CO_2. Blood samples were obtained from a heart puncture, and the *m. gastrocnemius*, *m. plantaris* and *m. soleus* were care- fully excised, weighed, frozen in isopentane pre- cooled with liquid nitrogen and stored at −80 °C until further analysis as described previously (Swaminathan et al. 2021).

IL-6

Serum IL-6 levels were assessed in blood samples obtained from puncturing the heart, using the enzyme-linked immunosorbent assay (Mouse IL-6 ELISA Kit, BMS603-2, Invitrogen, Thermo Fisher, Frederick, USA) and a spectrophotometric plate reader (Spark 10 M, Tecan Group Ltd, Zurich, Switzerland).

Preparation of muscle homogenates

About 5 – 10 mg of frozen *m. plantaris* was homogenised in 100 lL ice-cold lysis buffer (50 mM Tris–HCl, 1 mM ethylene diamine tetra acetic acid, 1 mM ethylene bis (oxyethylene nitrilo) tetra acetic acid, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM b-glycerophosphate, 1% Triton X-100, pH 7.0). The homogenate was kept overnight at −80 °C and centrifuged the next day at 13,000 g for 10 mins and the supernatant used for determination of total protein concentration (BCA1, Sigma-Aldrich), and further SDH activity and western blot analysis.

Succinate dehydrogenase (SDH) activity

The SDH activity was determined as described previously (Swaminathan et al. 2021; Kvedaras et al. 2020). Ten lL of the supernatant was added to 96-well plates which contained 90 lL reaction reagent (50 mM NaPi buffer (pH ¼ 7.4), 1 mM KCN, 0.06 mM 2,6-DCPIP, 0.2% (wt/vol) bovine serum albumin) and 10 lL 100 mM sodium succinate solution. The change in absorbance at 600 nm per minute was measured spec-trophotometrically (Spark 10 M, Tecan Group Ltd, Zurich, Switzerland).

Western blot—Akt and VEGF

Twenty micrograms of protein was separated on 10–12% SDS-PAGE and transferred to PVDF mem- branes. Membranes were blocked with 5% non-fat milk for 1 hour and then incubated overnight (4 °C) with the following antibodies: VEGF (1:1000; MA116629, Thermo Fischer, USA), Akt (1:1,000; 9272, Cell Signalling Technology, Danvers, MA) and Phospho-Akt (Thr308; 1:1000; 9275, Cell Signalling Technology, Danvers, MA). Horseradish peroxidase- conjugated anti-mouse (1:10,000; 31430, Thermo Fischer, USA), anti-rabbit (1:3000; ab6721, Abcam, Cambridge, UK) or anti-rabbit (1:2000; 7071, Cell Signalling Technology, Danvers, MA) secondary anti- bodies were used for chemiluminescent detection of VEGF, Akt and p-Akt, respectively. Membranes were scanned and quantified using the ImageJ software.

Total nicotinamide-adenine dinucleotide (NAD)

Total NAD was measured using the commercial NAD/NADH Quantification Kit (Sigma-Aldrich MAK037). Approximately 20 mg of *m. gastrocnemius* was washed with cold PBS and homogenised in 400 lL

NADH/NAD Extraction Buffer provided with the kit. The supernatant was used to measure total NAD in a 96-well plate at 450 nm (Spark 10 M, Tecan Group Ltd, Zurich, Switzerland).

Table 2. Body mass (BM) (g), muscle mass (mg) and % hypertrophy of the *m. plantaris* before and after 18 weeks on experimental diet of young and old mice.

	YOUNG						OLD					
	BM (g) Wk 0	BM (g) Wk 18	*m. gast* (mg)	*m. plantaris* (mg)	*m. soleus* (mg)	% hypertrophy	BM (g) Wk 0	BM (g) Wk 18	*m. gast* (mg)	*m. plantaris* (mg)	*m. soleus* (mg)	% hypertrophy
Control	30.1 ± 1.3	31.5 ± 0.6[f]	129 ± 3[e,f]	15.4 ± 0.6[e,f]	10.2 ± 0.3[f]	30.9 ± 6.7	35.8 ± 0.7	37.2 ± 1.3	121 ± 2[f]	16.4 ± 0.6[e,f]	10.2 ± 0.5[f,g]	11.1 ± 3.1[g]
MR	29.1 ± 0.5	27.3 ± 1.7[e,f]	108 ± 6[e,f]	12.6 ± 0.9[e,f]	9.7 ± 0.5[f]	39.4 ± 5.2	37.2 ± 1.2	28.2 ± 0.6[e,f]	94 ± 7[e,f,g,h]	10.7 ± 0.7[e,f]	8.0 ± 0.5[e,f,g]	64.7 ± 13.9[e,f,g]
HFD	31.4 ± 0.7	44.5 ± 2.1[e,f,g,h]	141 ± 4[e,f]	16.7 ± 1.3[e,f]	14.2 ± 0.5[e,f,g]	21.0 ± 5.9	39.0 ± 2.2	42.8 ± 2.6[e,f,g]	110 ± 2[e,f,g,h]	13.9 ± 1.1[e,f]	11.5 ± 0.4[e,f,g]	26.4 ± 8.6[g]
MR þ HFD	31.3 ± 0.6	29.0 ± 3.3[e,f]	109 ± 4[e,f]	12.2 ± 0.8[e,f]	10.5 ± 0.5[f]	20.4 ± 5.2	37.6 ± 2.3	29.9 ± 1.3[e,f]	101 ± 4[e,f,g]	13.0 ± 0.8[e,f]	8.8 ± 0.2[e,f]	21.7 ± 5.7[g]

m. gast: gastrocnemius muscle; [a, c, m, h, x]: significantly different from young, control, MR, HFD, MR þ HFD group respectively at p 0.041. [e]: significantly different from week 0 at p 0.004. Data is presented as mean ± SEM.

Experimental diets: control, methionine restricted (MR), high fat diet (HFD), or MR þ HFD diet.

Statistics

Data are presented as mean, min, max or mean ± SEM. The Shapiro-Wilk test was applied to test whether the data were normally distributed. As the plasma IL-6 concentrations and SDH activity data were not normally distributed they were log-trans- formed before statistical analysis. A two-way ANOVA was used to test for differences in body and muscle mass, total *m. gastrocnemius* NAD and plasma IL-6 concentrations, with age and diet as factors. A repeated-measures ANOVA with muscle as within factor, and age and diet as between factors was per- formed on Akt and VEGF levels, and the log-trans- formed p-Akt and SDH activity data. If there were significant interactions, ANOVA post-hoc tests were performed to locate the differences. Effects and inter- actions were considered significant at $p < 0.05$. All analyses were performed using IBM SPSS Version 26.

Results

Body and muscle mass

The data on body mass, muscle mass and percentage hypertrophy of the *m. plantaris* have been published previously (Swaminathan et al. 2021), but have been repeated here for completeness (Table 2). A HFD resulted in an increased body mass in young mice (p ::; 0.036) and there was a trend towards an increase in body mass in the old group (p ¼ 0.056). MR induced a decrease (p ¼ 0.018) in body mass.

Table 2 also shows the mass of control *m. gastro- cnemius*, *m. soleus* and control and hypertrophied *m. plantaris* in young and old mice on control, MR, HFD and MR þ HFD diets. The *m. gastrocnemius* and *m. soleus* mass was higher in young than old animals ($p < 0.001$). Mice on MR and MR þ HFD had a lower *m. gastrocnemius* mass compared to those fed a control and HFD diet ($p < 0.001$), irrespective of age. For the *m. soleus* there was an age * diet interaction ($p < 0.001$). It appeared that in both young and old mice, those on a HFD had a greater *m. soleus* mass than those on MR and MR þ HFD ($p < 0.001$), while only young animals on a HFD had a larger *m. soleus* mass those on a control diet ($p < 0.001$). In addition, only in the old group did the MR-fed mice have a lower *m. soleus* mass than control and HFD groups (p ::; 0.003). There was no significant difference in *m. plantaris* mass between young and old animals (p ¼ 0.255). Animals on a MR and MR þ HFD diets both had lower *m. plantaris* mass than the control and HFD groups (p ::; 0.005), irrespective of age.

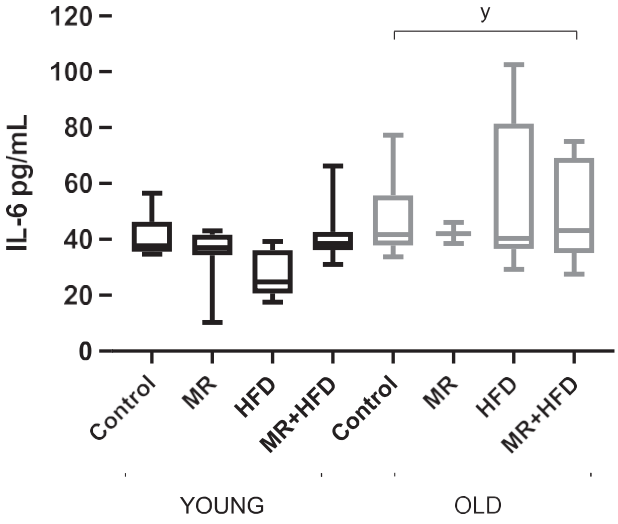
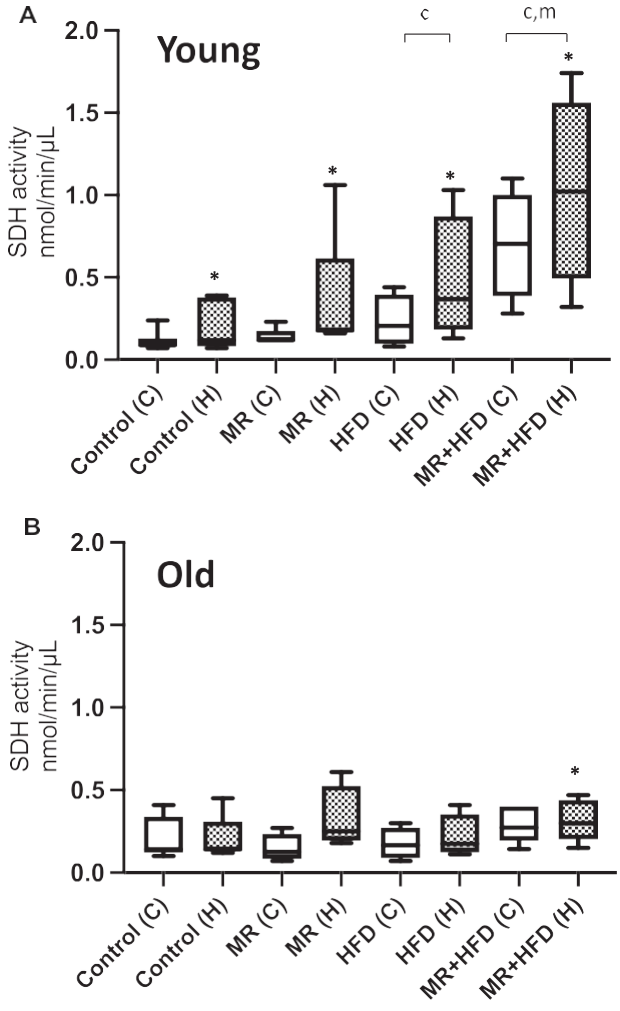


Figure 2. Plasma IL6 concentration in young (black boxes) and old (grey boxes) mice on a control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. *y*: significantly different from young at *p* ¼ 0.002. Data is presented as mean, min, max.

We did not observe a significant effect of diet on % hypertrophy in young mice (*p* ¼ 0.175). Old mice on MR, however, had a larger % hypertrophy as compared to the control, HFD and MR þ HFD groups (*p* ::; 0.027).



IL6

Figure 2 shows that old mice had higher serum IL-6 concentrations compared to young mice (p ¼ 0.002), with no significant effect of diet (p ¼ 0.079).

Figure 3. SDH activity in control (C—empty boxes) and hypertrophied (H—patterned boxes) *m. plantaris* in (A) young and (B) old mice on a control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. *: significantly different from diet-matched control leg at p ::; 0.015; c, m: significantly different from

SDH activity

Figure 3 shows that there was no main effect of age (p ¼ 0.075) on the SDH activity of the *m. plantaris*. There were main effects of diet and overload on the SDH activity (p < 0.001; Figure 2(A), B), as well as age * diet (p ¼ 0.011) and overload * diet (p ¼ 0.038) interactions. Further analyses revealed that the *m. plantaris* of young mice on a HFD or MR þ HFD diet had a higher SDH activity than those from mice on control diets, and those on a HFD þ MR had a greater SDH activity than those fed a MR diet (p ::; 0.047), irrespective of overload. In the old group, overload did induce an increase in the SDH activity (p ¼ 0.001), but such an overload-induced increase in SDH activity only occurred in animals on a MR þ HFD diet (p ¼ 0.007).

Akt, p-Akt and VEGF

There were no significant effects of age, diet, or over- load, nor any significant interactions for VEGF and Akt levels in the *m. plantaris* (Figures 4(A,B) and 5(A,B), respectively). The p-Akt was higher in *m. plantaris* from old than young mice (p ¼ 0.006) and in overloaded muscles, irrespective of age and diet (p < 0.001) (Figure 5(C,D)).

NAD

While there were no significant main effects of age or diet on the total NAD concentration in the *m. gastro-cnemius*, there was a significant age * diet interaction (p ¼ 0.048; Figure 6). Further analyses within the young (p ¼ 0.152) and old (p ¼ 0.159) groups separately did not reveal any significant differences between diets.

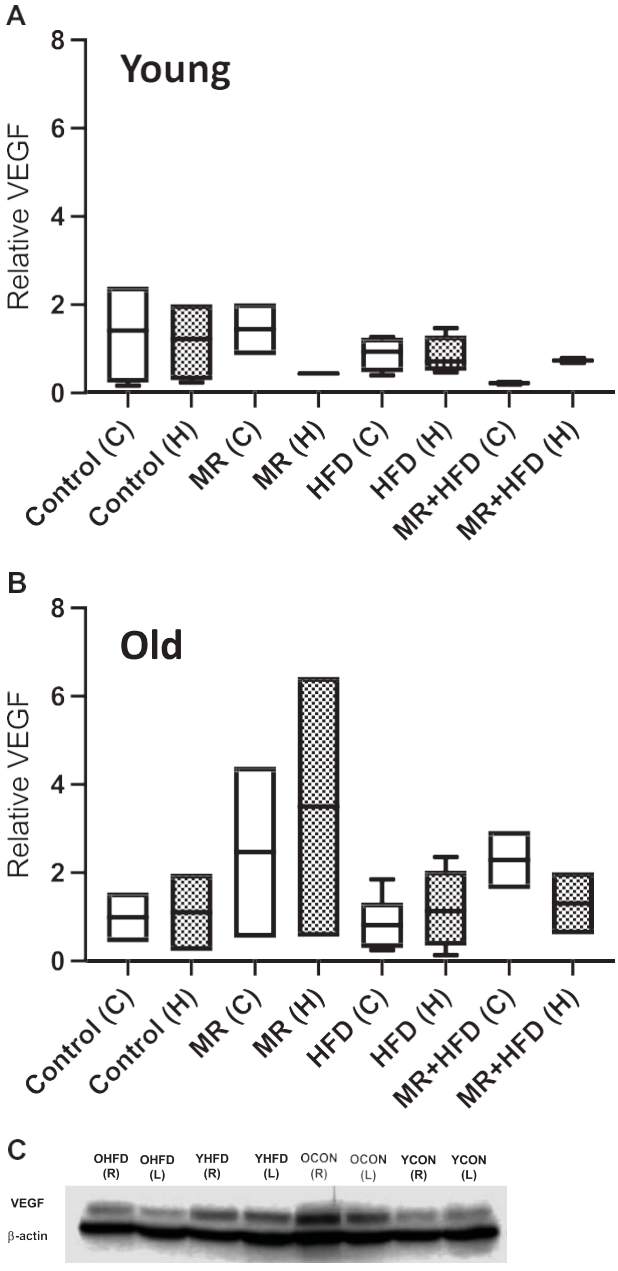


Figure 4. Relative VEGF in the control (C—empty boxes) and hypertrophied (H—patterned boxes) *m. plantaris* of (A) young and (B) old mice on a control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. Data is presented as mean, min, max. (C) Western blot images for VEGF and b-actin (as loading control) in control (L) and hypertrophied (R) *m. planta- ris* of old (O) and young (Y) mice on control and high fat diet (HFD).

Discussion

The main observation of the present study was that in contrast to our hypotheses, a high fat diet (HFD) did not accentuate, nor did methionine restriction (MR) alleviate the low-grade systemic inflammation in old mice. In addition, the MR-induced enhanced hypertrophic response in old mice (Swaminathan et al. 2021) was not associated with elevated levels of NAD in the muscle tissue, or elevated levels of VEGF or Akt in the hypertrophied muscles and the over- load-induced rise in p-Akt was independent of age or diet.

Low-grade systemic inflammation is often seen in old age (Visser et al. 2002; Bian et al. 2017; Schaap et al. 2006), and is reflected among others by an elevated circulating IL-6 concentration (Alberro et al. 2021; Hager et al. 1994)—something we observed also in our mice. The age-related rise in circulating IL-6 may be enhanced by a habitual intake of dietary fat as has been shown in postmenopausal women (Chmurzynska et al. 2019). We, however, did not see any further increase in the circulating IL-6 levels of old mice on a HFD,

which corresponds with other observations where a sustained HFD in ageing rats did not result in a further increase in IL-6 (Pongratz et al. 2015). We also did not see any significant impact of MR on circulating IL-6 levels in old mice, while others have seen a decrease in inflammation in adult 24-week-old mice (Sharma et al. 2019) and 22- week-old progeroid mice (Bárcena et al. 2018) after MR. It should be noted, however, that the inflammatory markers in those studies were measured in adipose tissue and liver, sites that are closely associated with metabolism of fat, and not in the circulation. While our data shows that there was an increase in circulating IL-6 concentrations in the old compared to young mice, it appears that there is not much of a decrease in MR-fed mice. Therefore, we can speculate that while MR does not significantly reduce age-associated inflammation, it certainly does not worsen the condition.

Although we did not observe an age-related decline in SDH activity (Proctor et al. 1995; Sczelecki et al. 2014), our data though not significant, indicate if any- thing, that the old control-fed mice have higher SDH activity than their younger counterparts, which is similar to earlier findings (Ballak et al. 2016).

In line with earlier work (Messa et al. 2020), we observed that young mice fed a HFD have a higher SDH activity than those on a control diet. It has been shown that a HFD may increase the SDH activity in muscle fibres from young, but not old mice (Messa et al. 2020). As we discussed previously, such a diminished responsiveness of the oxidative metabolism to a HFD in muscles from old mice may result in a lesser ability to oxidise fatty acids and consequently

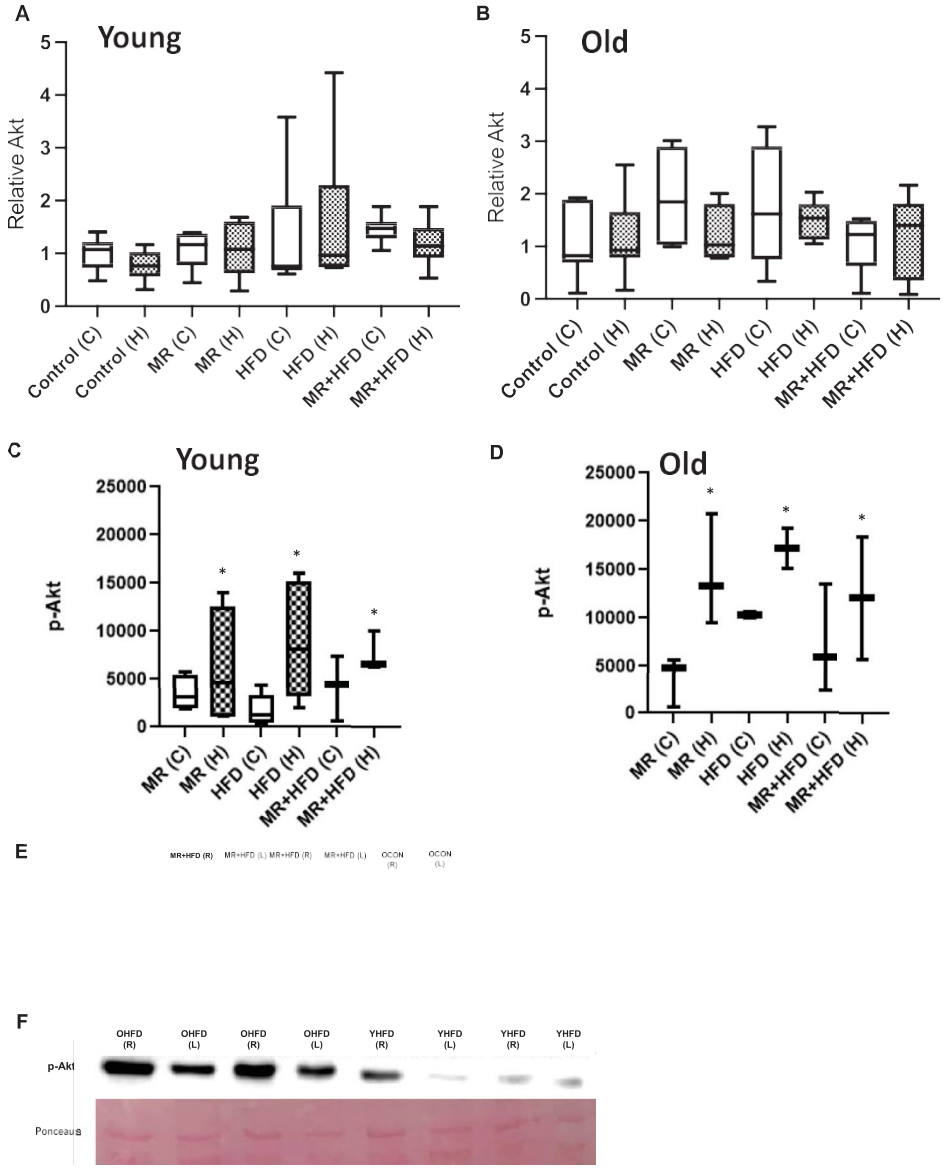


Figure 5. Relative Akt in the control (C—empty boxes) and hypertrophied (H—patterned boxes) *m. plantaris* of (A) young and (B)

old mice on a control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. p-Akt in the control (C—empty boxes) and hypertrophied (H—patterned boxes) *m. plantaris* of (C) young and (D) old mice on a methionine restricted MR, HFD and MR þ HFD. *: significantly different from diet-matched control leg at $p < 0.001$. Data is presented as mean, min, max. (E) Western blot images for Akt and b-actin (as loading control) in control (L) and hypertrophied (R) *m. plantaris* of old (O) and young (Y) mice on control and MR þ HFD. (F) Western blot images of p-Akt and corresponding Ponceau S in control (L) and hypertrophied (R) *m. plantaris* of old (O) and young (Y) mice on a HFD.

a more rapid intramyocellular lipid accumulation in old than young muscles (Degens, Swaminathan, and Tallis 2021). Here we extend this observation that also an overload-induced increase in SDH activity seen in young mice did not occur in old mice except—but to a much lesser extent than in young animals—if they were fed MR þ HFD. It thus appears that MR has only a limited impact on mitochondrial biogenesis in muscle, in contrast to the stimulation of mitochondrial biogenesis in brain (Naud,ı et al. 2007). We have no explanation for this discrepancy, but it may reflect that the effects of MR on oxidative metab- olism are tissue specific.

Although we did not observe an attenuated hyper- trophy or diminished increase in p-Akt of the *m. plantaris* in mice on a HFD, previous work in young mice has shown that a chronic HFD impaired the ability of the *m. plantaris* to hypertrophy in response to mechanical overload (Sitnick, Bodine, and Rutledge 2009). It has been proposed that a HFD- or obesity- induced inflammation may attenuate the hypertrophic response via e.g. a loss of the positive association

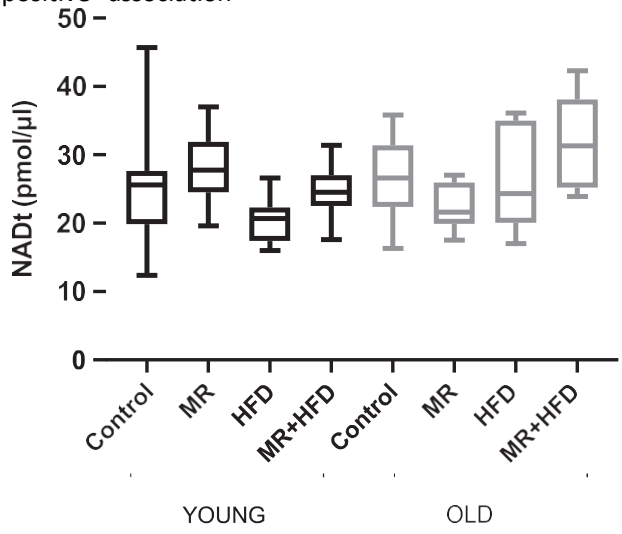


Figure 6. Total NAD concentration in young (black boxes) and old (grey boxes) mice on a control, methionine restricted (MR), high fat diet (HFD) and MR þ HFD. Data is presented as mean, min, max.

between insulin like growth factor-I (IGF-I) and muscle strength (Barbieri et al. 2003), thereby contributing to anabolic resistance (Rennie 2009). We, how- ever, did not see a HFD-induced rise in IL-6 corresponding with the absence of any HFD-induced attenuation of p-Akt expression and hypertrophic response in both young and old mice in our study. This corresponds with the observation that in young- obese adults there was no obesity-induced impairment of anabolic signalling (Hulston et al. 2018).

Previous studies have shown an attenuated hyper- trophic response in old mice that was associated with a reduced expression of the VEGF receptor Flk-1 (Ballak et al. 2016). The attenuated hypertrophy in old mice we observed was, however, not associated with lower muscle VEGF levels, and neither was VEGF elevated in the hypertrophied muscles.

Interestingly, the p-Akt levels were higher in the *m. plantaris* from old than you mice, corresponding with the higher IGF-I mRNA expression and regenerative drive in old than young muscle (Edström and Ulfhake 2005). This perhaps reflects that in older muscles there is an attempt, via upregulation of anabolic signalling pathways, to avert adverse skeletal muscle changes, even before an overt age-related loss of muscle mass occurs.

MR did enhance the hypertrophic response in old mice (Swaminathan et al. 2021), but here we show that this was no associated with an enhanced over- load-induced expression of p-Akt. Previously we speculated that this enhanced response might be related to VEGF-induced angiogenesis, as previous work showed that (1) the attenuated muscle hyper- trophy in old mice was associated with impaired angiogenesis (Hendrickse et al. 2021; Ballak et al. 2016) and (2) sulphur amino acid restriction (Longchamp et al. 2018) led to skeletal muscle angio- genesis in old mice. In contrast to this

expectation, we did not see a MR-induced rise in VEGF in the normal or overloaded *m. plantaris*. Another factor that may enhance angiogenesis is NAD via the activation of SIRT1 (Das et al. 2018), but we did not see an MR-induced rise in NAD. This corresponds with the absence of a beneficial effect of resveratrol—thought to act via SIRT1—on angiogenesis, Flk-1 expression and the hypertrophic response in old mice (Ballak et al. 2016). Perhaps the main underlying factor con- tributing to the MR-induced enhanced hypertrophic response in old mice was attributable to a reduction in low-grade systemic inflammation, as systemic inflammation may cause an attenuated hypertrophic response (Degens 2010). This then is not so much related to increased protein synthesis, as we did not observe any MR-induced increase in Akt or p-Akt, but perhaps more to alleviating some of the inflammation-induced protein breakdown.

MR does not appear to be detrimental to markers of muscle hypertrophy in old age, nor to reduce oxidative capacity and VEGF levels. A possible explanation for this absence of beneficial effects of MR on VEGF, is the age-at-onset of the dietary intervention. For instance, Edington, Cosmas, and McCafferty 1972 observed the existence of a "threshold age" in rats, beyond which there is no benefit to longevity to start exercise training. Similarly, it has been observed that the increase in life span elicited by sulphur amino acid restriction decreased the later in life the restriction was started (Nichenametla, Mattocks, and Malloy 2020). Additionally, it is difficult to distinguish between the direct, tissue-specific responses to MR and the responses observed in one anatomical site and modulated in another (Orgeron et al. 2014).

Future work may investigate to what extent MR affects muscle fibre type composition and to what extent an MR-induced reduction in inflammation in old age may underlie the MR-induced enhanced muscle hypertrophy, perhaps via reduction of protein breakdown.

In conclusion, MR enhanced the skeletal muscle hypertrophic response in old age that was accompanied with an increase in p-Akt without significant changes in muscle oxidative capacity, low-grade systemic inflammation, NAD, VEGF or Akt levels. Interestingly, MR did not rescue the blunted hypertrophic response in old mice on a HFD. If this work can be translated to human application, then MR bears promise to enhance interventions to increase muscle mass and strength in older people, who should be encouraged to lower excessive intake of fats.

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Disclosure statement

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