

Supraphysiological hyperinsulinaemia is necessary to stimulate skeletal muscle protein anabolism in older adults: evidence of a true age-related insulin resistance of muscle protein metabolism

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Received: 6 May 2009 / Accepted: 28 May 2009 / Published online: 9 July 2009
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Abstract

Aims/hypothesis The physiological increase in muscle protein anabolism induced by insulin is blunted in healthy, glucose-tolerant older adults. We hypothesised that the age-related defect in muscle protein anabolism is a true insulin resistance state and can be overridden by supraphysiological hyperinsulinaemia.

Methods We used dye dilution, stable isotopic and immunoblotting techniques to measure leg blood flow, muscle protein synthesis, protein kinase B/mammalian target of rapamycin (Akt/mTOR) signalling, and amino acid kinetics in 14 healthy, glucose-tolerant older volunteers at baseline, and during an insulin infusion at postprandial (PD, $0.15 \text{ mU min}^{-1} 100 \text{ ml}^{-1}$) or supraphysiologically high (HD, $0.30 \text{ mU min}^{-1} 100 \text{ ml}^{-1}$) doses.

Results Leg blood flow, muscle protein synthesis, and Akt/mTOR signalling were not different at baseline. During hyperinsulinaemia, leg blood flow ($p<0.01$) and muscle protein synthesis increased in the HD group only (PD [%/h]: from 0.063 ± 0.006 to 0.060 ± 0.005 ; HD [%/h]: from

0.061 ± 0.007 to 0.098 ± 0.007 ; $p<0.01$). Muscle Akt phosphorylation increased in both groups, but the increase tended to be greater in the HD group ($p=0.07$). The level of p70 ribosomal S6 kinase 1 (S6K1) phosphorylation increased in the HD group only ($p<0.05$). Net amino acid balance across the leg improved in both groups, but a net anabolic effect was observed only in the HD group ($p<0.05$).

Conclusions/interpretation We conclude that supraphysiological hyperinsulinaemia is necessary to stimulate muscle protein synthesis and anabolic signalling in healthy older individuals, suggesting the existence of a true age-related insulin resistance of muscle protein metabolism.

Keywords Ageing · Insulin · Insulin resistance · Muscle protein synthesis · Sarcopenia

Abbreviations

Akt	Protein kinase B
eEF2	Eukaryotic elongation factor 2
FSR	Fractional synthetic rate
ICG	Indocyanine green
mTOR	Mammalian target of rapamycin
S6K1	p70 ribosomal S6 kinase 1
USC	University of Southern California, Los Angeles
UTMB	University of Texas Medical Branch at Galveston
4E-BP1	4E binding protein 1

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Introduction

Physiological hyperinsulinaemia stimulates muscle protein synthesis and anabolism in young people regardless of

glucose tolerance as long as amino acid delivery to the muscle is not decreased [1–9]. However, ageing is associated with an inability of insulin to stimulate muscle protein synthesis and net amino acid uptake in healthy, glucose-tolerant persons [8, 10]. This defect is associated with endothelial dysfunction and a blunted mammalian target of rapamycin (mTOR) signalling, which limit muscle perfusion and protein anabolism during hyperinsulinaemic stimulation in older adults [8, 10, 11]. The decline in muscle protein anabolic response to insulin is likely to be responsible for the observed reduction in muscle protein anabolism following mixed feeding in older people [12, 13], and thus to contribute to the development of age-related muscle loss (sarcopenia). Sarcopenia, in turn, is an important contributor to frailty and disability associated with ageing [14], and it has recently been estimated that its healthcare cost may be comparable with that of osteoporosis [15].

We have recently demonstrated that an insulin-sensitising intervention, aerobic exercise, can acutely restore a normal protein anabolic response to physiological hyperinsulinaemia in older individuals with normal glucose tolerance [11], suggesting that this age-related defect shares at least some features with the classic insulin resistance of type 2 diabetes. However, the inability of muscle protein metabolism to mount an anabolic response during physiological hyperinsulinaemia in otherwise healthy older people cannot be defined as an insulin resistance state unless it is demonstrated that insulin concentrations above the physiological range can produce an anabolic effect on skeletal muscle.

The purpose of this study was to determine if exposure of skeletal muscle to insulin concentrations above the physiological postprandial range could induce a protein anabolic response in healthy older adults.

Methods

Participants We studied 14 older volunteers (11 men and three women) from the greater Galveston/Houston area and the Los Angeles metropolitan area. All individuals provided informed written consent before participating in the study, which was approved by the Institutional Review Boards of the University of Texas Medical Branch at Galveston (UTMB) and the University of Southern California, Los Angeles (USC).

All volunteers were healthy and independent (no impairment in the activities of daily living or instrumental activities of daily living), but were not engaged in an exercise training programme. Screening was performed with clinical history, physical examination and a number of laboratory tests including a 2 h 75 g oral glucose tolerance

test. Only individuals with screening results within normal limits were randomly assigned to one of two groups receiving either a postprandial-dose (PD) or high-dose (HD) insulin infusion. The characteristics of enrolled volunteers are summarised in Table 1. This experiment was part of a larger randomised study in which young and older individuals were assigned to various treatments. Data from six volunteers in the PD group have been included in a separate report [10]. Primary outcome data (blood flow and muscle protein synthesis) obtained in young, body mass index (BMI)-matched individuals ($n=7$, age 31 ± 2 years, BMI 26.2 ± 1.4 kg/m², $p=\text{NS}$ vs older volunteers by one-way ANOVA) undergoing the same postprandial insulin infusion experiment as the PD group are also reported.

Study design We measured blood flow, muscle protein, amino acid and glucose kinetics, and components of the protein kinase B/mammalian target of rapamycin (Akt/mTOR) signalling pathway in the post-absorptive basal state (0–5 h) and during insulin infusion (5–8 h). Each volunteer was admitted to the UTMB or USC General Clinical Research Center in the afternoon prior to the infusion study. At 18:30 hours, all volunteers were fed a standardised dinner (one-third of their estimated daily energy requirements: 60% carbohydrate, 20% protein and 20% fat). They were given a snack at 22:00 hours, after which they were allowed only water ad libitum until the end of the experiment. The next morning, catheters were inserted into a forearm vein for tracer and dextrose infusion, in a contralateral hand vein for arterialised blood sampling, and in the femoral artery and vein of one leg for blood sampling. The arterial catheter was also used for infusion of insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) and indocyanine green (ICG; Akorn, Buffalo Grove, IL, USA).

Table 1 Characteristics of the older volunteers undergoing a local insulin infusion in one leg at postprandial or high dose

Characteristic	Postprandial dose	High dose	<i>p</i> value
Sex: male/female	6/2	5/1	0.70
Age (years)	69±1	72±2	0.14
Weight (kg)	83±3	86±4	0.56
Height (cm)	176±4	168±3	0.14
BMI (kg/m ²)	27±1	30±1	0.06
Lean body mass (kg)	54±3	56±2	0.56
Fat mass (kg)	21±2	26±3	0.17
Leg volume (l)	9.3±0.4	9.0±0.7	0.63

Data are means±SE

At 07:30 hours, after drawing a blood sample to measure background phenylalanine enrichment and ICG concentration, a primed (2 $\mu\text{mol/kg}$) continuous infusion of L-[ring- $^{13}\text{C}_6$]phenylalanine (0.05 $\mu\text{mol kg}^{-1} \text{min}^{-1}$; Cambridge Isotope Laboratories, Andover, MA, USA) was started and maintained until the end of the experiment. At 2 h after initiation of the stable isotope infusion, we took the first muscle biopsy from the lateral portion of the vastus lateralis of the leg bearing the femoral catheters, using a 5 mm Bergström biopsy needle, aseptic procedure and local anaesthesia with 1% (wt/vol.) lidocaine injected subcutaneously and on the muscle fascia. The 150–400 mg muscle sample was quickly rinsed with ice-cold saline, blotted, and immediately frozen in liquid nitrogen and stored at -80°C until analysis. During the fifth hour of tracer infusion, ICG was infused in the femoral artery (0.5 mg/min); four blood samples were taken at 10 min intervals from the femoral and hand veins to measure ICG concentration, after which another series of four blood samples were taken from the femoral artery and vein to measure glucose concentrations and phenylalanine concentrations and enrichments. Four additional samples were also drawn from the hand and femoral veins to measure systemic and femoral insulin concentrations, respectively. A second muscle biopsy was taken at the end of the fifth hour as previously described.

Immediately after the second biopsy, an insulin infusion was started directly into the femoral artery at the following infusion rates: PD=0.15 $\text{mU min}^{-1} 100 \text{ ml}^{-1}$ ($\sim 0.2 \text{ mU kg}^{-1} \text{min}^{-1}$); HD=0.30 $\text{mU min}^{-1} 100 \text{ ml}^{-1}$ ($\sim 0.4 \text{ mU kg}^{-1} \text{min}^{-1}$). This technique allowed us to avoid the remarkable reduction in blood amino acid concentrations typically seen during systemic insulin infusion [1, 16]. A euglycaemic clamp was performed infusing 20% (wt/vol.) dextrose at a variable rate, which was adjusted based on blood glucose concentrations measured every 5–10 min.

During the eighth and last hour of the experiment, ICG was infused again to measure leg blood flow, and blood samples were taken to measure ICG concentrations, phenylalanine and glucose enrichments and concentrations, and insulin concentrations, as described above for the basal period. At 480 min, before stopping the insulin and tracer infusion, a third muscle biopsy was taken as described above.

Analytical methods Plasma glucose concentration was measured using an automated enzymatic method (YSI, Yellow Springs, OH, USA), and serum ICG concentration was measured spectrophotometrically at $\lambda=805 \text{ nm}$ (Beckman Coulter, Fullerton, CA, USA) [17, 18].

Blood phenylalanine concentrations and enrichments were determined by GC–MS (Agilent Technologies, Palo Alto, CA, USA) as previously described [19]. Phenylalanine concentration was used as a predictor of insulin-

induced changes in the concentration of all essential amino acids [20].

Free amino acids and proteins were extracted from muscle tissue as previously described [19], and intracellular free phenylalanine concentrations and enrichments were determined by GC–MS as previously described [19]. Mixed muscle protein-bound phenylalanine enrichment was analysed by GC–MS after protein hydrolysis and amino acid extraction [19] using the external standard curve approach [21].

Immunoblotting was performed on $\sim 50 \text{ mg}$ of frozen tissue after homogenisation [22]. Total protein concentration was determined using the Bradford assay (Smartspec Plus, BioRad, Hercules, CA, USA). The supernatant fraction was diluted (1:1) in a sample buffer mixture and then boiled for 3 min at 100°C , except for samples used to assay 4E binding protein 1 (4E-BP1). Samples were separated by electrophoresis (Criterion, BioRad); gels contained an internal loading control (rodent skeletal muscle) and molecular mass ladder (Precision Plus, BioRad). Proteins were then transferred to a polyvinylidene difluoride membrane (BioRad) and incubated with primary antibody overnight at 4°C . The next morning, blots were incubated with secondary antibody for 1 h. Chemiluminescent solution (ECL Plus; Amersham BioSciences, Piscataway, NJ, USA) was applied to each blot and optical density measurements were obtained (ChemiDoc, BioRad). Densitometric analysis was performed using Quantity One 4.5.2 software (BioRad). Membranes were stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA) and reprobed for total protein. Density values were normalised to the internal loading control and data were expressed as phospho divided by total protein. Primary antibodies were purchased from Cell Signalling (Beverly, MA, USA). Phospho-specific antibody concentrations were: 1:1,000 for Akt (Ser 473), mTOR (Ser 2448), 4E-BP1 (Thr $^{37/46}$), eukaryotic elongation factor 2 (eEF2) (Thr 56); and 1:500 for phospho-p70 ribosomal S6 kinase 1 (S6K1) (Thr 389). Antibody dilutions for all total proteins were 1:1,000. Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2,000).

Calculations Mixed muscle protein fractional synthetic rate (FSR) was calculated using the precursor-product model to calculate the synthesis rate as follows [23]:

$$\text{FSR} = (\Delta E_p/t) / [(E_{M(1)} + E_{M(2)})/2] \times 60 \times 100$$

where ΔE_p is the increment in protein-bound phenylalanine enrichment between two sequential biopsies, t is the time between the two sequential biopsies, $E_{M(1)}$ and $E_{M(2)}$ are

the phenylalanine enrichments in the free intracellular pool in the two sequential biopsies. Data are expressed as per cent per hour.

Muscle phenylalanine kinetic parameters were calculated using both the two- and the three-pool arteriovenous balance models as each model provides unique information regarding leg plasma (two-pool) and intracellular (three-pool) phenylalanine kinetics [1]. The models' assumptions and validation have been extensively reviewed [24]. The two- and three-pool model parameters were calculated as previously described [11]. Phenylalanine utilisation for protein synthesis and release from breakdown during hyperinsulinaemia were corrected by changes in the intracellular phenylalanine concentration as previously described [25].

Leg glucose utilisation was calculated as net glucose uptake across the leg:

$$\text{leg glucose uptake} = \text{BF} \times (C_A - C_V)$$

where BF is the blood flow, and C_A and C_V are the arterial and venous concentrations, respectively.

Changes in blood flow can affect insulin concentration during exogenous infusion. Moreover, insulin recycling from the systemic circulation can increase insulin delivery to the leg. Thus, we calculated the total insulin delivery rate to the leg [10] as follows:

$$\text{insulin delivery} = \text{Ins}_{\text{FV}} \times \text{BF}$$

We used the femoral insulin concentration (Ins_{FV}) in the calculation because arterial insulin concentration was not measurable as the arterial catheter was used for insulin infusion. Because some insulin is cleared by the muscle tissue [26], this method may slightly underestimate the total insulin delivery rate. Nonetheless it provides an additional means to evaluate the degree of muscle insulinisation.

Statistical analysis Statistical analysis was performed using SigmaStat v3.5. Primary outcome measures were blood flow and FSR. Sample size was calculated to detect between group differences of 50% and 20% in the change from baseline of FSR and blood flow, respectively, with $\alpha=0.05$ and $\beta=0.80$. The Kolmogorov–Smirnov test (with Lilliefors' correction) was used for normality testing. Volunteer's characteristics and baseline values for all measured variables were analysed using the Student's *t* test or the rank sum test when data failed the normality test. The effect of the insulin dose on the response variables was analysed using ANOVA for repeated measures, the main effects being group (PD, HD) and time (basal, insulin infusion). If the data failed the normality test, log transformation was performed before conducting ANOVA

in order to achieve reasonably normal distribution. Pairwise multiple comparisons were performed using the Tukey–Kramer test. Differences were considered significant at $p<0.05$, with a trend at $p<0.10$.

Results

Blood flow Leg blood flow was not different between groups at baseline and increased significantly only in the HD group ($p<0.01$) with a power of 0.86 (Fig. 1). The change in blood flow in the HD group was comparable with that observed in younger individuals during a postprandial insulin infusion.

Insulin and glucose Systemic and femoral vein insulin concentrations were not different at baseline; they were significantly increased in both groups during insulin infusion ($p<0.0001$), but the change was larger in the HD group ($p<0.05$) (Table 2). Insulin delivery to the leg was

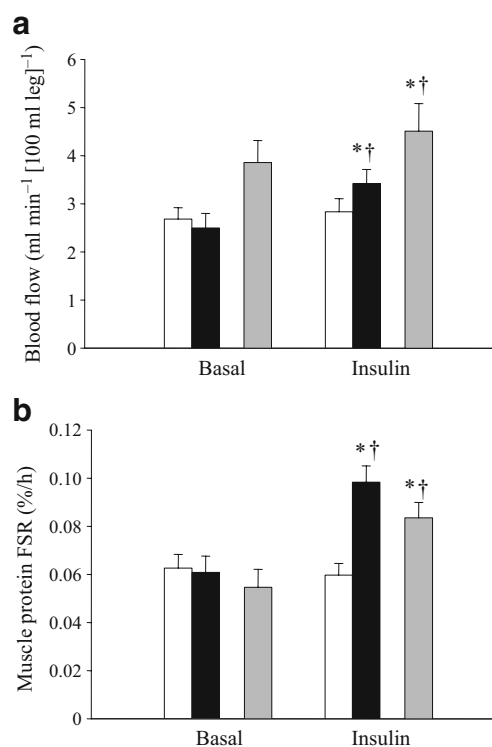


Fig. 1 Blood flow and muscle protein FSR in older volunteers at baseline and during the intra-arterial infusion of insulin at two different rates, which were chosen to increase the femoral vein insulin concentrations to either a postprandial level (PD; $n=8$; white bars) or a supraphysiologically high level (HD; $n=6$; black bars). Data from a group of younger BMI-matched individuals ($n=7$, age 31 ± 2 years), undergoing an insulin infusion at postprandial dose are also reported (grey bars). Values are the means \pm SE. * $p<0.05$ vs basal; $^\dagger p<0.01$ vs PD

Table 2 Insulin and glucose concentrations and leg kinetics in two groups of healthy older volunteers undergoing a local insulin infusion in one leg at postprandial or high dose

Measurement	Postprandial dose		High dose		ANOVA (<i>p</i> value)		
	Basal	Insulin	Basal	Insulin	Group	Time	Interaction
Insulin							
Systemic concentration (pmol/l)	53±9	149±23	37±9	190±21	0.57	<0.0001	0.07
Femoral vein concentration (pmol/l)	53±9	552±42	37±9	920±155	0.03	<0.0001	0.02
Delivery to the leg (pmol min ⁻¹ [100 ml leg] ⁻¹)	134±24	1,493±61	90±23	3,085±535	<0.01	<0.0001	<0.01
Glucose							
Arterial concentration (mmol/l)	4.8±0.2	4.6±0.2	5.2±0.2	5.0±0.2	0.28	0.18	0.54
Infusion rate (μmol kg ⁻¹ min ⁻¹)		11.5±1.0		16.9±1.3	<0.01		
Leg uptake (μmol min ⁻¹ [100 ml leg] ⁻¹)		1.8±0.4		3.5±0.5	0.01		

Data are means±SEM

not different between groups at baseline and it increased significantly during insulin infusion in both groups ($p<0.0001$), with a larger effect ($p<0.01$) in the HD group.

Plasma arterial glucose concentrations were not different between groups at baseline and did not change during insulin infusion with euglycaemic clamp. However, the exogenous glucose infusion rate and leg glucose uptake during insulin infusion were significantly higher in the HD group ($p<0.05$).

Phenylalanine concentrations and enrichments Phenylalanine concentrations in the artery, in the vein and in the muscle were not different at baseline, and were slightly but significantly decreased in both groups during insulin infusion ($p<0.05$), with no differences between groups (Table 3). Phenylalanine enrichments in the femoral artery and vein and in the muscle tissue were not different between groups in the basal period, and were at steady state during both sampling periods (data not shown). Phenylalanine enrichment in the femoral artery, vein and muscle increased slightly but significantly ($p<0.05$) during insulin infusion in both groups, with no group differences.

Mixed muscle FSR Mixed muscle protein FSR (%/h) was not different between groups in the basal state and during insulin infusion was significantly increased in the HD group only ($p<0.05$) with a power of 0.83 (Fig. 1). The change in mixed muscle FSR in the HD group was comparable with that observed in younger volunteers during a postprandial insulin infusion.

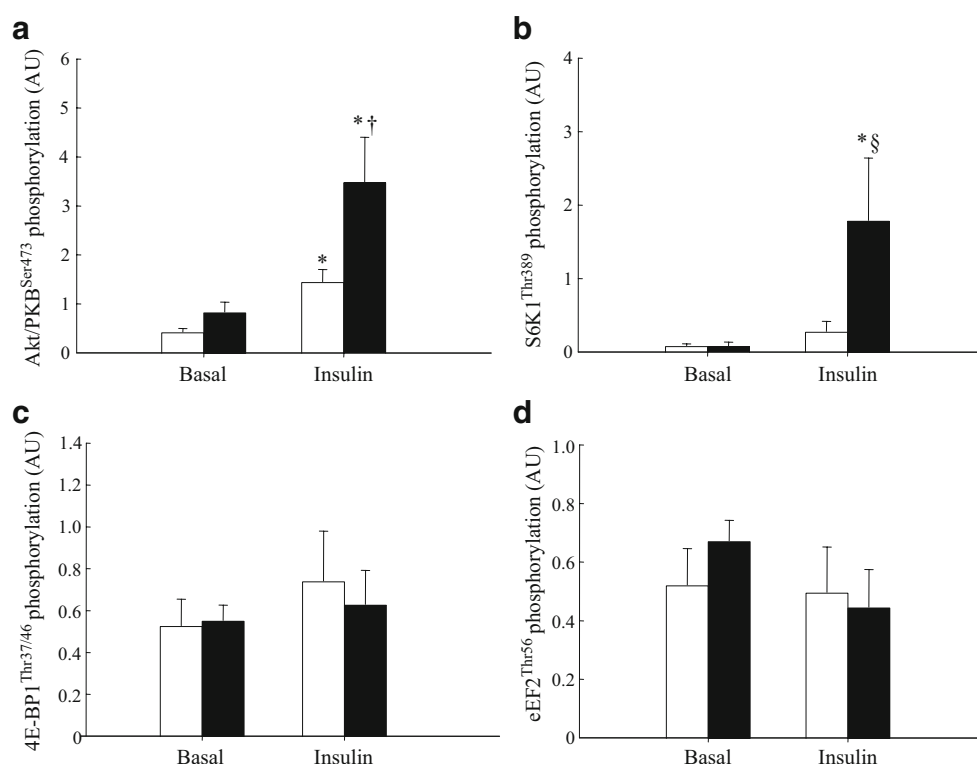
Akt and mTOR signalling For all five selected proteins, the baseline phosphorylation status was not different between groups and the total protein content did not change over time in either group. Akt/PKBSer⁴⁷³ phosphorylation increased significantly with insulin (time effect $p<0.01$), but the response tended to be greater in the HD group ($p=0.07$) (Fig. 2). mTOR^{Ser2448} phosphorylation did not increase with insulin ($p=0.12$) with no significant group effect or interaction. S6K1^{Thr389} phosphorylation increased significantly with insulin only in the HD group ($p<0.05$) with a significant difference between groups ($p<0.05$). 4EBP1^{Thr37/46} phosphorylation and eEF2^{Thr56} phosphorylation did not change with insulin in either group, with no difference between groups.

Table 3 Phenylalanine concentrations and enrichments at baseline and during insulin infusion in two groups of healthy older volunteers undergoing a local insulin infusion in one leg at postprandial or high dose

Phenylalanine	Postprandial dose		High dose		ANOVA (<i>p</i> value)		
	Basal	Insulin	Basal	Insulin	Group	Time	Interaction
Concentration (μmol/l)							
Femoral artery	60±2	55±1	61±3	57±4	0.68	<0.001	0.39
Femoral vein	66±2	55±1	68±3	55±4	0.81	<0.0001	0.12
Muscle tissue	88±6	66±6	81±5	72±4	0.90	<0.01	0.22
Enrichment (tracer/tracee ratio [%])							
Femoral artery	9.2±0.2	10.3±0.2	9.7±0.2	10.4±0.2	0.70	<0.001	0.33
Femoral vein	6.7±0.1	8.1±0.1	7.0±0.1	8.5±0.1	0.64	<0.01	0.77
Muscle tissue	5.2±0.3	6.2±0.3	5.9±0.4	6.7±0.4	0.27	<0.05	0.81

Data are means±SEM

Fig. 2 Phosphorylation of Akt/PKB, S6K1, 4E-BP1 and eEF2 in older volunteers at baseline and during the intra-arterial infusion of insulin at two different rates, chosen to increase the femoral vein insulin concentrations to either a postprandial level (PD: $n=6$ for Akt/PKB and S6K1; $n=5$ for 4E-BP1 and eEF2; white bars) or a supra-physiologically high level (HD: $n=6$ for all proteins; black bars). Values are the mean \pm SE. * $p<0.05$ vs basal; † $p=0.07$ vs PD; § $p<0.05$ vs PD. AU, absorbance unit



Phenylalanine kinetics None of the phenylalanine kinetic variables were significantly different between groups in the basal period (Table 4). With insulin infusion, phenylalanine delivery to and release from the leg increased in the HD group only ($p<0.05$). A significant interaction was found for phenylalanine release from the leg, which tended to increase in the HD group while decreasing from basal in the PD group.

There was a time effect ($p<0.05$) for phenylalanine release in blood from proteolysis (leg rate of appearance)

due to a significant decrease in the PD group ($p<0.05$) with no change in the HD group. In contrast, there was a trend ($p=0.06$) for a time by group interaction for phenylalanine disappearance from the blood (leg rate of disappearance), an index of protein synthesis, because of a significant increase in the HD group only ($p<0.05$).

Phenylalanine transport into the muscle free pool and intracellular availability did not change significantly with insulin, whereas phenylalanine transport from the muscle free pool into the venous blood decreased in both groups ($p<0.05$).

Table 4 Phenylalanine kinetics at baseline and during insulin infusion in two groups of healthy older volunteers undergoing a local insulin infusion in one leg at postprandial or high dose

Measurement ^a	Postprandial dose		High dose		ANOVA		
	Basal	Insulin	Basal	Insulin	Group	Time	Interaction
Delivery to the leg	160 \pm 12	154 \pm 12	150 \pm 17	195 \pm 21	0.39	0.03	0.02
Release from the leg	175 \pm 13	155 \pm 11	167 \pm 16	186 \pm 20	0.50	0.95	0.05
Net balance across the leg	-15 \pm 2	-1 \pm 3	-17 \pm 2	9 \pm 4	0.17	<0.0001	0.04
Release in blood from proteolysis	62 \pm 8	46 \pm 8	55 \pm 3	46 \pm 7	0.80	<0.01	0.47
Disappearance from the blood	46 \pm 8	45 \pm 8	38 \pm 5	55 \pm 7	0.88	0.10	0.06
Transport into muscle free pool	115 \pm 17	88 \pm 15	103 \pm 10	115 \pm 20	0.45	0.80	0.26
Transport from muscle free pool	130 \pm 18	88 \pm 13	120 \pm 8	106 \pm 22	0.70	0.02	0.23
Release from muscle proteolysis	70 \pm 8	61 \pm 12	62 \pm 5	61 \pm 10	0.76	0.50	0.57
Utilisation for muscle protein synthesis	55 \pm 7	59 \pm 11	45 \pm 7	71 \pm 11	0.94	0.04	0.05
Intracellular availability	185 \pm 23	148 \pm 23	166 \pm 12	176 \pm 26	0.75	0.48	0.16

Data are means \pm SEM. ^aUnits are nmol min⁻¹ (100 ml leg)⁻¹

Phenylalanine release from muscle proteolysis did not change significantly with insulin in either group. There was a significant time effect and time by group interaction for phenylalanine utilisation for muscle protein synthesis, which increased significantly during insulin infusion only in the HD group ($p < 0.05$). Net phenylalanine balance across the leg improved in both groups in response to insulin ($p < 0.0001$), but the increase was significantly greater in HD such that net balance became positive only in the HD group ($p < 0.01$), indicating net muscle protein anabolism.

Discussion

The results of this study indicate for the first time that skeletal muscle protein metabolism is truly insulin resistant in healthy, glucose-tolerant older adults. Specifically, we found in healthy older persons that supraphysiological hyperinsulinaemia was necessary to stimulate mixed muscle protein synthesis and net muscle protein anabolism while, consistent with previously published data [8, 10, 11], hyperinsulinaemia within the physiological postprandial range had only minor effects on muscle protein metabolism. Improvements in muscle protein anabolism observed with supraphysiological hyperinsulinaemia were associated with enhanced amino acid delivery, induced by increased leg blood flow, and enhanced Akt/PKB and S6K1 signalling. When we compare the effects of the two insulin doses on protein synthesis and blood flow in our elderly individuals with those obtained in BMI-matched healthy younger individuals with physiological hyperinsulinaemia (Fig. 1), it is clear that healthy older adults require double the insulin dose in order to increase muscle protein synthesis and vasodilatation to levels normally achieved by younger individuals during postprandial hyperinsulinaemia. A fascinating aspect of these results is that despite some analogies with the classic insulin resistance of glucose metabolism as observed in type 2 diabetes, the mechanisms underlying insulin resistance of muscle protein metabolism with ageing are probably different.

First, our volunteers, although overweight, had a normal glucose tolerance as assessed at screening, and exhibited a leg glucose uptake proportional to the insulin dose and consistent with uptakes previously reported in younger healthy individuals under comparable experimental conditions [1, 10]. Moreover, while volunteers in the HD group tended to be heavier than those in the PD group, they responded positively to insulin. Thus, if adiposity had a negative impact on the muscle protein anabolic response to insulin, it would have led to underestimation of the effect of the high insulin dose on skeletal muscle protein anabolism. Furthermore, we have previously shown that type 2 diabetes and excess adiposity do not influence the acute

response of skeletal muscle protein synthesis and anabolism in younger individuals [9], and we report here that blood flow and muscle protein synthesis are stimulated by a postprandial insulin dose in younger overweight individuals. Others have reported that the degree of glucose control does not affect skeletal muscle protein synthesis in type 2 diabetic patients [27]. Thus, it is becoming apparent that the age-related insulin resistance of muscle proteins and the classic insulin resistance of glucose metabolism leading to type 2 diabetes are two independent conditions, although they may share some characteristics.

Second, these results and others [1, 10, 11] suggest that the vasodilatory effect of insulin plays a much larger role in the modulation and stimulation of muscle protein anabolism in young and older individuals compared with its effect on glucose metabolism, for which it appears to be rate limiting only when glucose uptake is elevated and the artery-to-vein glucose concentration gradient is high [28–30]. An increase in microvascular recruitment increases the amount of tissue perfused and amino acid delivery to the muscle even in the presence of small decreases in amino acid concentrations, as occurred in the HD group of this study and in previous studies [1, 10, 11]. This interplay between insulin vasodilatation and amino acid availability appears to be an essential component of the overall anabolic effect of insulin. In fact, when profound hypoaminoacidaemia is induced with a systemic insulin infusion in the absence of amino acid replacement [7, 20, 31–33], amino acid delivery to the muscle will be reduced and the insulin anabolic effect will be mainly ascribed to reduced protein breakdown, rather than an increase in synthesis. However, if amino acids concentrations are maintained during a systemic insulin infusion, then insulin has a protein anabolic effect on human skeletal muscle [4, 6–8, 33, 34]. On the other hand, if amino acid concentrations are greatly increased to high postprandial levels using mixtures containing large amounts of arginine, the anabolic effect of insulin on protein synthesis may be masked by significant amino acid-induced increases in blood flow [35]. Consistent with previous reports [10, 11, 36], we found that physiological elevations in blood insulin were unable to increase blood flow in elderly individuals. However, we also found that supraphysiological hyperinsulinaemia induced a significant increase in blood flow, and this effect was associated with stimulation of muscle protein synthesis and net anabolism. It has been shown that the first effect of insulin is a rapid, nitric oxide-dependent microvascular recruitment that precedes increases in total blood flow and the effects on intracellular signalling and glucose uptake in muscle [37]. Although we did not directly measure capillary recruitment, it is likely that the differential effects of the two insulin doses on total leg blood flow were accompanied by concomitant differential

effects on muscle perfusion, with the higher dose being the only one able to stimulate microvascular recruitment. It is thus conceivable that the age-related endothelial dysfunction may limit the insulin-induced increase in nutrient flow, amino acid uptake and, consequently, muscle protein synthesis. Impaired endothelial-dependent capillary recruitment may also be responsible for the suboptimal anabolic response to mixed feeding in older persons [12], as lack of vasodilation reduces the amount of muscle tissue perfused and, consequently, nutrient and hormone delivery. On the other hand, perfusion alone may not be the only limiting factor. Recent data suggest that insulin transport across the endothelium into the muscle is a saturable process requiring intact insulin signalling, and it can therefore be an important regulator of insulin availability and action [26, 38].

Third, the muscle cell signalling data also suggest a relative insulin resistance, which may be related to both endothelial dysfunction and anabolic resistance to the insulin stimulus. While the group receiving the postprandial insulin dose was able to increase Akt/PKB^{Ser473} phosphorylation (although to a lesser extent than the group receiving the supraphysiological insulin dose), it was incapable of increasing the phosphorylation and, presumably, the activity of downstream effectors known to regulate translation initiation and protein synthesis in skeletal muscle. In particular, in our older volunteers supraphysiological hyperinsulinaemia was capable of increasing the phosphorylation state of S6K1^{Thr389} whereas, consistent with previous reports [8, 11], insulin concentrations in the normal physiological range did not. It should be kept in mind that the phosphorylation status of our selected proteins was measured at baseline and again 3 h after the initiation of the insulin clamp. Therefore, because physiological hyperinsulinaemia increased Akt/PKB^{Ser473} phosphorylation, it is possible that phosphorylation of mTOR^{Ser2448} and S6K1^{Thr389} increased to some extent at earlier time points during the insulin clamp, but had returned to baseline values when we obtained the last muscle sample. On the other hand, supraphysiological hyperinsulinaemia in the older volunteers induced a much greater increase in Akt/PKB^{Ser473} phosphorylation, thus resulting in a sustained phosphorylation of S6K1^{Thr389} and a significant increase in the rate of muscle protein synthesis. This is consistent with the notion that supraphysiological hyperinsulinaemia is capable of overriding the insulin resistance of protein metabolism in older individuals.

While these results are very exciting and may add another important piece of understanding to the complex sarcopenia puzzle, generalisation to the entire population should be done cautiously. The complexity and invasiveness of the methods used to measure muscle protein metabolism is limiting with regards to sample size. For

example, only three older women were included in this study. It is still unclear whether sex has a significant effect on muscle protein turnover in young and older adults, since some studies have reported baseline differences [39, 40] while others show no effect [41–44]. Nonetheless, a recent study suggests that there is no sexual dimorphism in the response of muscle proteins to insulin [44]. Thus, while with the current data we cannot draw conclusions regarding the effect of sex on the muscle protein response to insulin in older people, we believe that our results obtained from men and women are valid.

In conclusion, our data provide for the first time evidence that skeletal muscle protein metabolism is insulin resistant in healthy, non-diabetic older persons, as protein anabolism can be elicited only by supraphysiological hyperinsulinaemia. This defect appears unrelated to glucose tolerance and associated with endothelial dysfunction and blunted anabolic signalling, although the relative importance of these two complex mechanisms is still unclear and warrants further investigation. Insulin resistance of muscle protein metabolism with ageing may be a contributor to the development of sarcopenia in older adults as it may decrease the physiological muscle anabolic response to feeding [12], thereby inducing a slow but progressive decline in muscle protein content. Future mechanistic studies will be necessary to clearly determine the role and contribution of insulin resistance and endothelial dysfunction to muscle loss in older people.

Acknowledgements We wish to thank the study volunteers, M. Zheng for superb technical assistance and J. D. Cadenas and the nurses and personnel of the UTMB and USC General Clinical Research Centers for their assistance with the clinical conduct of this study. This study was supported by grants no. R01 AG18311 and the UTMB Claude D. Pepper Older Americans Independence Center no. P30 AG024832 from the National Institute on Aging, National Institutes of Health (NIH); grant no. S10 RR16650 from the Shared Instrumentation Grant Program, National Center for Research Resources, NIH; General Clinical Research Center grants no. M01 RR00043 (USC) and M01 RR00073 (UTMB), and grant no. S10 RR16650 from the National Center for Research Resources, NIH.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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