



Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia–hyperaminoacidaemia in healthy young and middle-aged men and women

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A B S T R A C T

Increased dietary LC n –3PUFA (long-chain n –3 polyunsaturated fatty acid) intake stimulates muscle protein anabolism in individuals who experience muscle loss due to aging or cancer cachexia. However, it is not known whether LC n –3PUFAs elicit similar anabolic effects in healthy individuals. To answer this question, we evaluated the effect of 8 weeks of LC n –3PUFA supplementation (4 g of Lovaza®/day) in nine 25–45-year-old healthy subjects on the rate of muscle protein synthesis (by using stable isotope-labelled tracer techniques) and the activation (phosphorylation) of elements of the mTOR (mammalian target of rapamycin)/p70S6K (p70 S6 kinase) signalling pathway during basal post-absorptive conditions and during a hyperinsulinaemic–hyperaminoacidaemic clamp. We also measured the concentrations of protein, RNA and DNA in muscle to obtain indices of the protein synthetic capacity, translational efficiency and cell size. Neither the basal muscle protein fractional synthesis rate nor basal signalling element phosphorylation changed in response to LC n –3PUFA supplementation, but the anabolic response to insulin and amino acid infusion was greater after LC n –3PUFA [i.e. the muscle protein fractional synthesis rate during insulin and amino acid infusion increased from 0.062 ± 0.004 to 0.083 ± 0.007 %/h and the phospho-mTOR (Ser²⁴⁴⁸) and phospho-p70S6K (Thr³⁸⁹) levels increased by ~50%; all $P < 0.05$]. In addition, the muscle protein concentration and the protein/DNA ratio (i.e. muscle cell size) were both greater ($P < 0.05$) after LC n –3PUFA supplementation. We conclude that LC n –3PUFAs have anabolic properties in healthy young and middle-aged adults.

INTRODUCTION

LC n –3PUFAs [long-chain n –3 PUFAs (polyunsaturated fatty acids)] are essential nutrients with many

potential health benefits. The general consensus appears to be that LC n –3PUFAs, particularly EPA (eicosapentaenoic acid; C_{20:5, n-3}) and DHA (docosahexaenoic acid; C_{22:6, n-3}), have anti-inflammatory properties [1]

Key words: fish oil, mammalian target of rapamycin (mTOR), muscle protein synthesis, n –3 polyunsaturated fatty acid (PUFA), p70 S6 kinase (p70S6K).

Abbreviations: BSA, body surface area; CRP, C-reactive protein; DHA, docosahexaenoic acid; eEF2, eukaryotic elongation factor 2; EPA, eicosapentaenoic acid; FFM, fat-free mass; FSR, fractional synthesis rate; IL-6, interleukin-6; mTOR, mammalian target of rapamycin; MUFA, mono-unsaturated fatty acid; p70S6K, p70 S6 kinase; PUFA, polyunsaturated fatty acid; LC n –3PUFA, long-chain n –3 PUFA; SFA, saturated fatty acid; t-BDMS, t-butylidimethylsilyl; TNF- α , tumour necrosis factor- α ; TTR, tracer/tracee ratio.

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and reduce the risk of cardiovascular disease [1]. Furthermore, there is good evidence from studies in animals that LCn-3PUFAs improve the sensitivity of whole-body and muscle glucose metabolism to insulin [2-5], although the results from studies in human subjects are equivocal (reviewed by Fedor and Kelley [6]). There is also emerging evidence for a muscle anabolic effect of LCn-3PUFAs. For example, low-dose LCn-3PUFA supplementation (i.e. 1-2% of total daily energy intake, as in our present study), alone or in combination with amino acid supplementation, has been reported to help maintain whole-body protein synthesis, whole-body protein net balance and muscle mass in burned rats and tumour-bearing mice [7,8]. Furthermore, we have recently demonstrated [9] that LCn-3PUFA supplementation (4 g of Lovaza[®]/day) in older adults (≥ 65 years of age) significantly increased the rate of muscle protein synthesis during hyperinsulinaemia-hyperaminoacidaemia, most likely because of greater activation of the mTOR (mammalian target of rapamycin)/p70S6K (p70S6 kinase) signalling pathway, and Ryan et al. [10] reported that increased LCn-3PUFA intake blunted the loss of total body and limb FFMs (fat-free masses) in patients with resectable non-metastatic oesophageal cancer undergoing oesophageal cancer surgery. The exact mechanisms responsible for the beneficial effect of LCn-3PUFAs on muscle protein metabolism are unknown, but one might speculate that they are related to the anti-inflammatory properties of LCn-3PUFAs [1], because burn injury, cancer and aging are all associated with increased inflammatory activity [11-14], which is known to induce muscle loss [15,16]. On the other hand, it is possible that LCn-3PUFAs have intrinsic muscle protein anabolic properties, in which case they should also stimulate muscle protein synthesis in healthy young subjects. In fact, feed enriched in menhaden oil, a fish oil rich in EPA and DHA, doubled the insulin-stimulated non-oxidative whole-body disposal of amino acids (a marker of increased whole-body protein synthesis) and increased the activation of the mTOR/p70S6K signalling pathway in muscle of young and still-growing steers [5], suggesting this may be the case. The effect of LCn-3PUFA intake on muscle protein metabolism in healthy young adults, however, has not been studied to date. The purpose of the present study therefore was to determine the effect of LCn-3PUFA supplementation for 8 weeks on indices of muscle protein anabolism in human muscle in young/middle-aged adults. To this end, we measured the fractional rate of muscle protein synthesis (by using stable isotope-labelled tracer techniques) during basal post-absorptive conditions and during hyperinsulinaemia-hyperaminoacidaemia (within the range normally seen after meal consumption [17,18]), the concentrations of protein, RNA and DNA in muscle (to obtain indices of the protein synthetic capacity, translational

efficiency [19,20] and cell size [21]), and the activation (as phosphorylation) of elements of intracellular signalling pathways involved in the regulation of muscle protein synthesis [Akt, mTOR, p70S6K and eEF2 (eukaryotic elongation factor 2)] [22,23] in healthy men and women. We also measured markers of inflammation in plasma [CRP (C-reactive protein), IL-6 (interleukin-6) and TNF- α (tumour necrosis factor α)], the rate of appearance of glucose into plasma (an index of endogenous glucose production) and the rate of whole-body glucose uptake (glucose rate of disappearance) to gauge the relationship between the effect of LCn-3PUFAs on glucose and muscle protein metabolism.

MATERIALS AND METHODS

Subjects

Nine healthy individuals [five men and four women; age, 39.7 ± 1.7 years; BMI (body mass index), 25.9 ± 1.0 kg/m²; body fat determined by dual X-ray absorptiometry, 25 ± 3 %; values are means \pm S.E.M.] participated in the present study. All subjects were considered to be in good health after completing a comprehensive medical evaluation, which included a history and physical examination and standard blood tests. None of the subjects engaged in regular physical activities (i.e. they exercised ≤ 1.5 h/week), consumed fish oil supplements or took any medication; none reported excessive alcohol intake or consumed tobacco products. Written informed consent was obtained from all subjects before their participation in the study, which was approved by the Human Subjects Research Protection Office and the Clinical Research Unit Advisory Committee at Washington University School of Medicine in St Louis, MO, U.S.A.

Experimental protocol

Each subject completed two stable isotope-labelled tracer infusion studies to determine the effect of LCn-3PUFA supplementation on the rate of muscle protein synthesis and anabolic signalling during basal post-absorptive conditions and during insulin and amino acid infusion. The first study was performed within 1-3 weeks of screening (before the intervention); the second one took place after 8 weeks of dietary supplementation with 4 g of Lovaza[®]/day (GlaxoSmithKline) containing 1.86 and 1.50 g/day of the ethylesters of EPA and DHA respectively. Compliance was evaluated by pill count and changes in the muscle phospholipid fatty acid composition. We gave each subject an excess number of pills and asked them to return any remaining pills at the end of the study.

Before each muscle protein metabolism study, subjects were instructed to adhere to their usual diet and to refrain from vigorous physical activities for 3 days. The

evening before the study, subjects were admitted to the Clinical Research Unit at Washington University School of Medicine. At 20.00 h, they consumed a standard meal providing 50.2 kJ/kg of body weight (15% as protein, 55% as carbohydrates and 30% as fat). Subjects then rested in bed and fasted (except for water) until completion of the study the next day. At ~06.00 h on the following morning, a cannula was inserted into an antecubital vein for the infusion of stable isotope-labelled tracers (i.e. a phenylalanine tracer to measure the rate of muscle protein synthesis and a glucose tracer to measure the glucose rate of appearance in the systemic circulation); another cannula was inserted into a vein of the contralateral hand, which was warmed to 55 °C for blood sampling. At ~08.00 h, primed constant infusions of [*ring*-²H₅]phenylalanine (priming dose, 2.8 μmol · kg⁻¹ of FFM; infusion rate, 0.08 μmol · kg⁻¹ of FFM · min⁻¹) and [6,6-²H₂]glucose (priming dose, 18 μmol · kg⁻¹ of body weight; infusion rate, 0.22 μmol · kg⁻¹ of body · min⁻¹), all purchased from Cambridge Isotope Laboratories, were started and maintained for 7 h. A 4 h after the start of the tracer infusions, a hyperinsulinaemic–hyperaminoacidaemic clamp was started and maintained for 3 h. Human insulin (Novolin R; Novo Nordisk) was infused at a rate of 20 m-units · m⁻² BSA (body surface area) · min⁻¹ (initiated with two priming doses of 80 m-units · m⁻² BSA · min⁻¹ for 5 min and then 40 m-units · m⁻² BSA · min⁻¹ for an additional 5 min). Plasma amino acid availability was increased by providing an intravenous amino acid mixture (10% Travasol; Baxter) at a rate of 105 mg of amino acids · kg⁻¹ of FFM · h⁻¹ (priming dose, 35 mg of amino acids/kg of FFM). Euglycaemia (blood glucose concentration of ~5.5 mM) was maintained during the clamp procedure by variable rate infusion of 20% dextrose (Baxter) enriched to 2.5% with [6,6-²H₂]glucose. To adjust for the increased plasma amino acid availability and reduced hepatic glucose production during the clamp procedure, the [*ring*-²H₅]phenylalanine infusion rate was increased to 0.12 μmol · kg⁻¹ of FFM · min⁻¹ and the [6,6-²H₂]glucose infusion rate was decreased to 0.11 μmol · kg⁻¹ of body weight · min⁻¹.

Blood samples (4 ml) were obtained before beginning the tracer infusions, and then at 60, 90, 180, 210, 220, 230, 240, 270, 300, 330, 360, 390, 400, 410, and 420 min to determine the labelling of phenylalanine and glucose in plasma and plasma substrate, hormone and cytokine concentrations. Additional blood (~1 ml) was obtained every 10 min during the clamp to monitor plasma glucose concentration. Muscle tissue (~100 mg) was obtained under local anaesthesia (2% lidocaine) from the quadriceps femoris by using a Tilley-Henkel forceps [24] at 60 and 240 min to determine the basal rate of muscle protein synthesis (labelled phenylalanine incorporation into muscle protein; see the Calculations subsection) and the basal concentrations of phosphorylated elements

of intramuscular signal transduction proteins (Akt, mTOR, p70S6K and eEF2) involved in the regulation of muscle protein synthesis. A third muscle biopsy was obtained at 420 min (i.e. 3 h after starting the clamp procedure) to determine both the rate of muscle protein synthesis and the intracellular signalling responses to hyperinsulinaemia–hyperaminoacidaemia. The second and third biopsies were obtained from the same incision on the leg contralateral to that biopsied first; the forceps were directed in a proximal and distal direction so that the two biopsies were collected ~5–10 cm apart.

Sample processing and analyses

Blood (4 ml) was collected in pre-chilled tubes containing heparin, plasma was separated immediately by centrifugation and the glucose concentration was measured immediately. The remaining blood (~3 ml) was collected in pre-chilled tubes containing EDTA, plasma was separated by centrifugation within 30 min of collection and was then stored at –80 °C until final analyses. Muscle samples were rinsed in ice-cold saline immediately after collection, cleared of visible fat and connective tissue, frozen in liquid nitrogen and stored at –80 °C until final analysis.

Plasma glucose concentration was measured on an automated glucose analyser (Yellow Spring Instruments). Plasma insulin concentration was determined by RIA (Linco Research). Commercially available ELISA kits (R&D Systems) were used to determine plasma concentrations of CRP, TNF-α and IL-6.

Muscle phospholipid fatty acid composition was determined after extracting lipids from ~30 mg of muscle tissue with 2 ml of chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Phospholipids were then isolated using TLC (Whatman TLC LK 6D; Fischer Scientific), fatty acids were converted into their methyl esters by reacting with 10% acetyl chloride in methanol and their peak areas were measured by using GC–MS (MSD 5973 System; Hewlett-Packard).

To determine the labelling of plasma glucose, plasma proteins were precipitated with ice-cold acetone and hexane was used to extract plasma lipids. The aqueous phase, containing glucose, was dried by speed-vac centrifugation (Savant Instruments), glucose was derivatized with heptafluorobutyric acid and the TTR (tracer-to-tracee ratio) was determined using GC–MS (Hewlett-Packard MSD 5973 system with capillary column) as described previously [25].

To determine the plasma concentrations of phenylalanine and leucine (thought to be a major regulator of muscle protein synthesis [26]) and the labelling of phenylalanine in plasma, known amounts of [1-¹³C]phenylalanine and norleucine were added to an aliquot of each plasma sample, plasma proteins were precipitated and the supernatant, containing free amino acids, was collected to prepare the t-BDMS (t-butyldimethylsilyl) derivatives

of phenylalanine and leucine to determine their TTRs by GC-MS (MSD 5973 System; Hewlett-Packard) [27,28]. To determine phenylalanine labelling in muscle proteins and in tissue fluid, samples (~20 mg) were homogenized in 1 ml of trichloroacetic acid solution (3%, w/v), proteins were precipitated by centrifugation and the supernatant, containing free amino acids, was collected. The pellet containing muscle proteins was washed and then hydrolysed in 6 M HCl at 110°C for 24 h. Amino acids in the protein hydrolysate and supernatant samples were purified on cation-exchange columns (Dowex 50W-X8-200; Bio-Rad Laboratories), and the t-BDMS derivative of phenylalanine was prepared to determine its TTR by GC-MS (MSD 5973 System; Hewlett-Packard) [27,28]. The extent of phenylalanine labelling in plasma, muscle tissue fluid and muscle protein was calculated based on the simultaneously measured TTR of standards of known isotope labelling.

Western blot analysis was used to measure the phosphorylation of Akt, mTOR, p70S6K and eEF2. Briefly, frozen muscle tissue (~20 mg) was rapidly homogenized in ice-cold buffer [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM glycerophosphate, 50 mM sodium fluoride, 0.1% Triton X100 and 0.1% 2-mercaptoethanol containing one protease and one phosphatase inhibitor tablet (Roche Diagnostics)] at 10 µl per mg of tissue. Proteins were extracted by shaking for 15 min at 4°C, samples were then centrifuged at 13 000 g for 10 min at 4°C and the supernatant, containing the proteins, was collected. The protein concentration in the supernatant was determined by the Bradford method with a commercial reagent (B6916; Sigma-Aldrich) and adjusted to 3 mg/ml in 3 × Laemmli buffer. Protein (50 µg) from each sample was loaded on to 12% XT-Bis Tris gels, separated by SDS/PAGE and transferred on ice at 100 V for 45 min to methanol pre-wetted 0.2 µm PVDF membranes. Blots were then incubated sequentially with 5% (w/v) non-fat milk for 1 h, primary antibodies overnight at 4°C and then a secondary antibody (1:2000 anti-rabbit; New England Biolabs) for 1 h. The following primary antibodies were used at a dilution of 1:2000: anti-phospho-Akt (Thr³⁰⁸), anti-phospho-mTOR (Ser²⁴⁴⁸), anti-phospho-p70S6K (Thr³⁸⁹) and anti-phospho-eEF2 (Thr⁵⁶), and an anti-pan-actin (as a loading control), all purchased from New England Biolabs. Membranes were developed using Immobilon Western Chemiluminescent HRP substrate (Millipore), and the protein bands were visualized and quantified by densitometry on a Chemidoc XRS (Bio-Rad Laboratories) ensuring no pixel saturation. Results are expressed in relation to GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

To determine the muscle protein concentration (per mg of wet weight), the protein/DNA ratio (an estimate of cell size), the RNA/protein ratio (an index of the ribosomal capacity for protein synthesis) and the RNA/DNA

ratio (the cell capacity for protein synthesis) in muscle, tissue (~15 mg) was snipped before homogenization in 0.2 M HClO₄. After centrifugation at 2800 g and washing with 0.2 M HClO₄, the pellet was resuspended in 0.3 M NaOH to quantify alkali-soluble proteins using the Bradford assay and spectrophotometry. Next, proteins were precipitated with 1 M HClO₄ before centrifugation and removal of the supernatant for RNA quantification by spectrophotometry. The remaining pellet was resuspended in 2 M HClO₄ and incubated at 70°C for 1 h before centrifugation and removal of the supernatant for DNA quantification by spectrophotometry.

Calculations

The FSR (fractional synthesis rate) of mixed muscle protein was calculated from the rate of incorporation of [*ring*-²H₅]phenylalanine into muscle protein, using a standard precursor-product model as follows: $FSR = \Delta E_p / E_{ic} \times 1/t \times 100$ where ΔE_p is the change between two consecutive biopsies in extent of labelling (TTR) of protein-bound phenylalanine, E_{ic} is the mean labelling over time of the precursor for protein synthesis, and t is the time between biopsies. The free phenylalanine labelling in muscle tissue fluid was chosen to represent the immediate precursor for muscle protein synthesis (i.e. aminoacyl-t-RNA) [29]. In addition, we calculated the muscle protein FSR by using the average plasma phenylalanine enrichments between 60 and 240 min (basal) and between 270 and 420 min (clamp).

The translation efficiency (mg of protein produced · µg⁻¹ of RNA · h⁻¹) was calculated by dividing the product of the muscle protein FSR (in %/h) and the muscle protein concentration (in mg/g of wet tissue) by the muscle total RNA concentration (in µg/g of wet tissue) [19,20].

Glucose rate of appearance in plasma during basal conditions and the clamp procedure was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 30 min of the basal period and the last 30 min of the clamp. Glucose rate of appearance during basal conditions equals glucose rate of disappearance and represents endogenous glucose production, whereas, during the clamp procedure, glucose rate of appearance represents the sum of endogenous glucose production and the rate of infused glucose. The endogenous glucose production rate during the clamp was therefore calculated by subtracting the glucose infusion rate from the glucose rate of appearance; glucose rate of disappearance was assumed to be equal to the glucose rate of appearance plus the tracer infusion rate.

Statistical analysis

All data sets were tested for normality and skewed data sets were log-transformed for statistical analysis.

Differences before and after LCn – 3PUFA supplementation in single time-point measurements (e.g. systemic inflammatory markers and muscle phospholipid fatty acid composition) were evaluated by using Student's *t* tests. Repeated measures ANOVA and Tukey's post-hoc procedure were used to evaluate differences before and after LCn – 3PUFA supplementation in plasma glucose, insulin, phenylalanine and leucine concentrations, mixed muscle protein FSR and muscle intracellular signalling elements during basal post-absorptive conditions and during the clamp procedure. A *P* value of ≤ 0.05 was considered statistically significant. Results are presented as means \pm S.E.M. or medians (25th and 75th percentiles) for skewed data sets.

RESULTS

Compliance with LCn – 3PUFA supplementation and muscle phospholipid fatty acid composition

All subjects consumed ≥ 160 of the 224 pills assigned to them. Average compliance, as judged by the left-over pill count, was $94 \pm 3\%$. This was confirmed by analysis of the muscle phospholipid fatty acid profile, which demonstrated a doubling of the proportion of LCn – 3PUFAs at the expense of *n* – 6 PUFAs and MUFAs (mono-unsaturated fatty acids) with no change in SFA (saturated fatty acid) concentrations (Table 1).

Plasma substrate, insulin and cytokine concentrations

Plasma glucose, insulin, phenylalanine and leucine concentrations were not affected by LCn – 3PUFA supplementation, neither during basal post-absorptive conditions nor during the hyperinsulinaemic–hyperaminoacidaemic clamp (Table 2 and Supplementary Table S1 at <http://www.clinsci.org/cs/121/cs1210267add.htm>). During the clamp, the plasma glucose concentration was successfully maintained at ~ 5.4 mM and plasma insulin, phenylalanine and leucine concentrations increased ~ 4 -fold, 80% and 40% above basal values respectively (all $P < 0.001$) both before and after LCn – 3PUFA supplementation (Table 2).

As expected, the concentration of inflammatory markers in plasma was low in this healthy cohort of subjects and there were no differences (all $P \geq 0.33$) before and after LCn – 3PUFA supplementation in the plasma concentrations of CRP [0.61 (0.39, 1.03) compared with 0.73 (0.17, 1.23) mg/l respectively], TNF- α (0.30 \pm 0.02 compared with 0.31 \pm 0.02 pg/ml respectively) and IL-6 [1.49 (1.05, 3.25) compared with 1.34 (0.98, 1.47) pg/ml respectively].

Table 1 Muscle phospholipid fatty acid profile before and after 8 weeks of LCn – 3PUFA supplementation

Values are means \pm S.E.M.

Fatty acid	Percentage of total fatty acids		<i>P</i> value
	Before	After	
SFA			
C _{14:0}	0.48 \pm 0.05	0.42 \pm 0.03	
C _{16:0}	17.39 \pm 0.53	17.69 \pm 0.48	
C _{18:0}	18.35 \pm 0.77	19.82 \pm 0.70	
Total	36.22 \pm 0.76	37.93 \pm 0.96	0.21
MUFA			
C _{16:1, n-7}	0.58 \pm 0.15	0.39 \pm 0.05	
C _{18:1, n-9}	8.21 \pm 1.13	6.35 \pm 0.44	
Total	8.79 \pm 1.27	6.74 \pm 0.48	0.08
<i>n</i> – 6 PUFA			
C _{18:2, n-6}	32.09 \pm 0.79	29.98 \pm 0.71	
C _{20:3, n-6}	1.33 \pm 0.10	1.27 \pm 0.10	
C _{20:4, n-6}	17.19 \pm 0.73	15.16 \pm 0.48	
Total	50.61 \pm 0.95	46.41 \pm 0.59	0.01
<i>n</i> – 3 PUFA			
C _{20:5, n-3}	0.66 \pm 0.11	2.57 \pm 0.31	
C _{22:5, n-3}	1.81 \pm 0.09	2.30 \pm 0.08	
C _{22:6, n-3}	1.91 \pm 0.17	4.05 \pm 0.35	
Total	4.38 \pm 0.33	8.93 \pm 0.67	<0.001

Plasma phenylalanine and glucose and muscle free phenylalanine enrichments

Plasma phenylalanine TTR was steady between 60 and 420 min, and plasma glucose TTR was steady between 210 and 240 min of the basal period and 390 and 420 min during the hyperinsulinaemic–hyperaminoacidaemic clamp, both before and after LCn – 3PUFA supplementation (Supplementary Table S1). The muscle free phenylalanine enrichments before and after LCn – 3PUFA supplementation were 0.066 ± 0.004 and 0.062 ± 0.004 respectively at the end of the basal post-absorptive period and 0.074 ± 0.003 and 0.072 ± 0.004 respectively at the end of the hyperinsulinaemic–hyperaminoacidaemic clamp.

Muscle protein concentration and synthesis

Both the alkali-soluble protein concentration and the protein/DNA ratio, a measure of cell size [21], increased ($P \leq 0.04$) after LCn – 3PUFA supplementation (Figure 1). However, neither the muscle RNA concentration (0.62 ± 0.07 compared with 0.68 ± 0.04 μ g of RNA/mg of muscle wet weight; $P = 0.45$) nor the RNA/protein ratio (5.8 ± 0.9 compared with 5.3 ± 0.5 μ g of RNA/mg of protein; $P = 0.65$), an index of the ribosomal capacity for protein synthesis, in muscle were affected by LCn – 3PUFA supplementation. There was a trend for an increase in the RNA/DNA ratio (Figure 1), the cell

Table 2 Plasma glucose, insulin, leucine and phenylalanine concentrations during basal post-absorptive conditions and during the hyperinsulinaemic–hyperaminoacidaemic clamp procedure before and after 8 weeks of LCn–3PUFA supplementation

Values are means \pm S.E.M. ^a $P < 0.001$ compared with the corresponding basal value.

Plasma measurement	Before		After	
	Basal	Clamp	Basal	Clamp
Glucose (mM)	4.9 \pm 0.1	5.4 \pm 0.1 ^a	4.9 \pm 0.1	5.4 \pm 0.1 ^a
Insulin (μ -units/ml)	5.2 \pm 0.8	28.9 \pm 1.8 ^a	5.6 \pm 0.7	31.4 \pm 2.2 ^a
Phenylalanine (μ M)	56 \pm 5	98 \pm 8 ^a	56 \pm 3	100 \pm 6 ^a
Leucine (μ M)	113 \pm 5	162 \pm 7 ^a	113 \pm 5	165 \pm 9 ^a

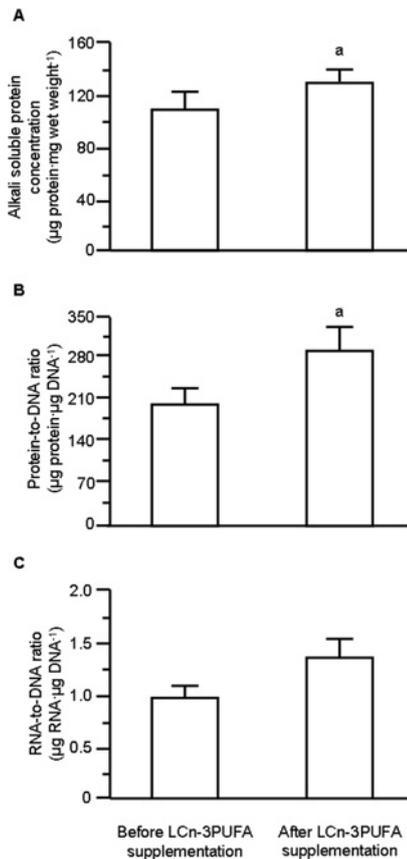


Figure 1 Muscle protein concentration, cell size and capacity for protein synthesis

Muscle alkali-soluble protein concentration (A), the protein/DNA ratio in muscle (an index of cell size) (B), and the RNA/DNA ratio (an index for the cell capacity for protein synthesis) (C) before and after 8 weeks of LCn–3PUFA supplementation. Values are means \pm S.E.M. ^a $P < 0.05$ compared with before LCn–3PUFA supplementation. The difference in the RNA/DNA ratio did not reach statistical significance ($P = 0.13$).

capacity for protein synthesis, but the difference did not reach statistical significance ($P = 0.13$).

The basal muscle protein FSR (calculated by using the muscle free phenylalanine enrichment as the precursor enrichment) was not different before and after

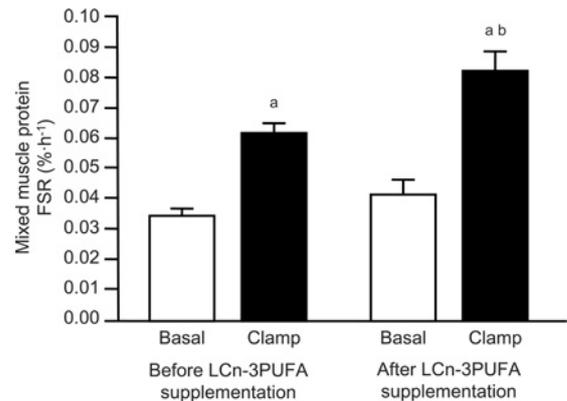


Figure 2 Muscle protein FSRs

Muscle protein FSRs during basal post-absorptive conditions and during the hyperinsulinaemic–hyperaminoacidaemic clamp procedure before and after 8 weeks of LCn–3PUFA supplementation. Values are means \pm S.E.M. ANOVA revealed a significant main effect of hyperinsulinaemia–hyperaminoacidaemia ($P < 0.001$) and a hyperinsulinaemia–hyperaminoacidaemia–LCn–3PUFA interaction ($P = 0.01$). ^a $P < 0.01$ compared with the corresponding basal value; ^b $P < 0.01$ compared with the corresponding value before LCn–3PUFA supplementation.

LCn–3PUFA supplementation (Figure 2). Insulin and amino acid infusion led to a marked increase in the muscle protein FSR ($P < 0.001$) and the anabolic response (i.e. the increase from basal values) was $\sim 50\%$ greater after LCn–3PUFA supplementation ($0.042 \pm 0.005\%$ compared with $0.027 \pm 0.005\%/h$; $P = 0.01$). Consequently, the muscle protein FSR during insulin and amino acid infusion was significantly greater ($P < 0.01$) after than before LCn–3PUFA supplementation (Figure 2). Using the plasma phenylalanine enrichment as the precursor enrichment in the muscle protein FSR calculation did not affect the results. The basal muscle protein FSR was not different before and after LCn–3PUFA supplementation ($0.022 \pm 0.002\%$ compared with $0.025 \pm 0.003\%/h$ respectively; $P = 0.18$). Insulin and amino acid infusion led to a marked increase in the muscle protein FSR ($P < 0.001$) and the anabolic response (i.e. the increase from basal values) was $\sim 50\%$ greater after LCn–3PUFA supplementation (0.038 ± 0.005 compared with $0.025 \pm 0.004\%/h$; $P = 0.04$).

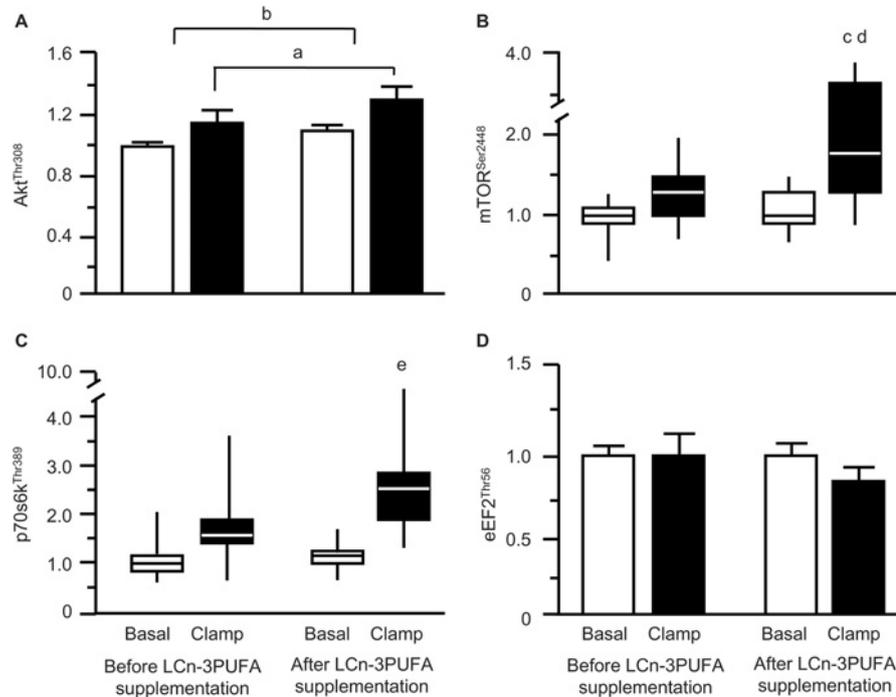


Figure 3 Skeletal muscle anabolic signalling responses

Levels (in arbitrary units) of phosphorylated Akt (Thr³⁰⁸) (A), mTOR (Ser²⁴⁴⁸) (B), p70S6K (Thr³⁸⁹) (C) and eEF2 (Thr⁵⁶) (D) during basal post-absorptive conditions and during the hyperinsulinaemic–hyperaminoacidaemic clamp procedure before and after 8 weeks of LCN-3PUFA supplementation. Values are means \pm S.E.M., or medians (horizontal lines) with quartiles (boxes) and minimum and maximum values (vertical lines). ^{a,b}For Akt (Thr³⁰⁸), ANOVA revealed a main effect of hyperinsulinaemia–hyperaminoacidaemia ($P = 0.042$) and LCN-3PUFA ($P = 0.023$) but no interaction ($P = 0.976$). For mTOR (Ser²⁴⁴⁸) and p70S6K (Thr³⁸⁹), ANOVA revealed a main effect of hyperinsulinaemia–hyperaminoacidaemia ($P < 0.01$) and a significant interaction ($P < 0.05$). Tukey post-hoc analysis located the specific differences as follows: ^c $P < 0.01$ compared with the corresponding basal value; ^d $P < 0.05$ compared with the corresponding value before LCN-3PUFA supplementation; ^e $P < 0.05$ compared with the corresponding basal value.

LCN-3PUFA supplementation did not affect the translational efficiency during basal conditions (0.0068 ± 0.0008 compared with 0.0079 ± 0.0009 mg of protein $\cdot \mu\text{g}^{-1}$ of RNA $\cdot \text{h}^{-1}$ before and after supplementation respectively; $P = 0.42$); however, during insulin/amino acid infusion, the translational efficiency was $\sim 35\%$ greater after than before LCN-3PUFA supplementation (0.0234 ± 0.0018 compared with 0.0174 ± 0.0012 mg of protein $\cdot \mu\text{g}^{-1}$ of RNA $\cdot \text{h}^{-1}$ respectively; $P < 0.01$).

Phosphorylation of anabolic signalling transduction proteins

The level of Akt (Thr³⁰⁸) in muscle was greater during insulin/amino acid infusion than during basal conditions ($P = 0.042$ for the main effect of clamp) and was greater (although the difference was small) after than before LCN-3PUFA supplementation ($P = 0.023$). There was, however, no difference ($P = 0.976$) before and after LCN-3PUFA supplementation in the extent of the hyperinsulinaemia–hyperaminoacidaemia-induced increase in Akt (Thr³⁰⁸) phosphorylation

(Figure 3A). The basal concentrations of mTOR (Ser²⁴⁴⁸) and p70S6K (Thr³⁸⁹) in muscle were not different before and after LCN-3PUFA supplementation; ANOVA revealed a significant stimulatory effect of hyperinsulinaemia–hyperaminoacidaemia ($P < 0.01$ for the main effect of clamp) and a significant hyperinsulinaemia–hyperaminoacidaemia–time (pre-post intervention) interaction ($P < 0.05$). Tukey's post-hoc analysis indicated that this was due to an increase in mTOR (Ser²⁴⁴⁸) and p70S6K (Thr³⁸⁹) activation after LCN-3PUFA supplementation (Figures 3B and 3C). Neither hyperinsulinaemia–hyperaminoacidaemia ($P = 0.27$) nor LCN-3PUFA supplementation ($P = 0.21$) had an effect on the level of eEF2 (Thr⁵⁶) in muscle (Figure 3D).

Glucose kinetics

LCN-3PUFA supplementation had no effect on whole-body glucose kinetics. Basal glucose rate of appearance was $9.4 \pm 0.3 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ before and $9.4 \pm 0.3 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ after LCN-3PUFA supplementation ($P = 0.90$). During

the clamp, endogenous glucose rate of appearance decreased by $\sim 70\%$ from basal values ($P < 0.001$) to $2.7 \pm 0.4 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ before and to $2.6 \pm 0.5 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ after LCn-3PUFA supplementation (no difference in the extent of decrease before and after supplementation, $P = 0.74$). Glucose rate of disappearance during the clamp was $25.9 \pm 2.3 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ before and $27.4 \pm 2.8 \mu\text{mol} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$ after LCn-3PUFA supplementation ($P = 0.46$).

DISCUSSION

In the present study, we provide evidence that LCn-3PUFA supplementation causes a considerable increase in the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged adults. These findings complement and extend the results we have recently obtained in older adults [9] and demonstrate that LCn-3PUFA supplementation not only alleviates the muscle protein anabolic resistance associated with old age [9,30-32], but can actually boost the anabolic response to nutritional stimuli in healthy muscle from young and middle-aged adults.

The specific mechanism(s) by which LCn-3PUFAs act on the muscle protein synthesis process remain mostly unknown. Our present results indicate that LCn-3PUFAs alone are not sufficient to elicit an anabolic effect (because the basal rate of muscle protein synthesis was not affected by LCn-3PUFA supplementation), but that they require additional anabolic stimuli such as amino acids and augment their anabolic effect by increasing the activation of the mTOR/p70S6K signalling pathway (which is considered an integral control point for muscle protein anabolism [33] and muscle cell growth [34-36]) and translational efficiency. What caused the greater activation of the mTOR/p70S6K signalling pathway after LCn-3PUFA supplementation remains unclear. It most probably was not increased Akt signalling because, although LCn-3PUFAs increased the level of Akt (Thr³⁰⁸), the increase occurred both during basal conditions and hyperinsulinaemia-hyperaminoacidaemia, whereas the stimulatory effect of hyperinsulinaemia-hyperaminoacadaemia was the same before and after LCn-3PUFA supplementation. Thus the effect was probably mediated via one or more alternative pathway(s) which have yet to be determined (but may include, for example, Rheb or vps34 [37,38]). Considering the observed changes in skeletal muscle phospholipid composition, it is also possible that LCn-3PUFA supplementation modulated key substrates along the anabolic signalling cascades by affecting membrane lipid composition and/or fluidity [39,40]. For example,

increased membrane DHA content activates PKC (protein kinase C) [39], which stimulates translational activity [41]. It is unlikely that the beneficial effect of LCn-3PUFAs on muscle protein synthesis was related to their anti-inflammatory properties [42,43] because our subjects were young and healthy and we did not detect any treatment-induced changes in inflammatory cytokine concentrations in plasma, most likely because the concentrations were very low to begin with.

The increases in Akt, mTOR and p70S6K phosphorylation during the hyperinsulinaemic-hyperaminoacidaemic clamp in our present study were small and did not always reach statistical significance at the $P < 0.05$ level before intervention. This was most probably the result of a type 2 error associated with Tukey's post-hoc analysis; in fact, when we applied a Student's *t*-test to evaluate the clamp-induced increases in mTOR and p70S6K phosphorylation before LCn-3PUFA supplementation, we obtained *P* values of 0.01 and 0.06 respectively. It is unlikely that the small increase in signalling activation was due to the timing of the muscle biopsy (i.e. 3 h after the start of the insulin, amino acid and glucose infusion) because the phosphorylation of IRS-1 (insulin receptor substrate-1), PI3K (phosphoinositide 3-kinase), Akt and mTOR *in vivo* in human muscle increases quickly and then remains steady (and elevated above basal values) for at least 180 min during constant insulin infusion and increased amino acid delivery to the muscle [44-46]. The small increases in anabolic signalling element phosphorylation were most probably due to the relatively low insulin and amino acid infusion rate which we chose to avoid a potential 'ceiling effect'. Specifically, we infused amino acids and insulin at rates close to those used to achieve the half-maximal amino acid-induced increase in muscle protein synthesis [47] and insulin-mediated increase in Akt phosphorylation [48]. In fact, our results are well in line with the results obtained by other investigators [31], who found that, during low-dose insulin infusion (similar to the one used in the present study) in conjunction with a high dose amino acid infusion (double the one used in this protocol), Akt, mTOR and p70S6K phosphorylation increased by $\sim 30\%$, $\sim 30\%$ and $\sim 90\%$ respectively. The respective values in our present study were $\sim 25\%$, $\sim 25\%$ and $\sim 50\%$. Furthermore, although it may seem as if there was a dissociation between mTOR and p70S6K activation, because the magnitude of change in the phosphorylation of the two appeared to be different, the apparent discrepancy in signal activation is not surprising. The activation of mTOR and downstream signalling is known to be complex involving not only phosphorylation, but also the regulation of interactions with many of its binding partners such as PRAS40 (proline-rich Akt substrate of 40 kDa),

RAPTOR (regulatory associated protein of mTOR) and DEPTOR [15,49,50]. Furthermore, although previous work showed that p70S6K was downstream of mTOR, it is now known that mTOR and p70S6K phosphorylate one another and the phosphorylation of mTOR on Ser²⁴⁴⁸ is mediated by p70S6K [51]. Thus one cannot expect a simple 1:1 relationship in the extent of mTOR and p70S6K phosphorylation. However, we can be fairly sure on the basis of our present results that mTOR/p70S6K signalling was increased by LCn-3PUFA supplementation.

We made our measurements of muscle protein synthesis during a 3-h infusion of insulin, amino acids and glucose because the rate of muscle protein synthesis rises quickly (within <30 min) in response to increased amino acid availability but then returns to basal values after ~2.5–3.0 h [52]. Therefore we assume that the increase in the anabolic response after LCn-3PUFA supplementation was due to an increase in the magnitude of the anabolic response. However, we cannot rule out the possibility that the effect was due to an increase in the duration of the anabolic effect of nutritional stimuli. Similarly, it is possible, but unlikely, that the greater mTOR and p70S6K phosphorylation after LCn-3PUFA supplementation was due to prolonged activation rather than greater peak magnitude of activation because, as pointed out above, the phosphorylation of anabolic signalling elements increases quickly and then remains steady (and elevated above basal values) for at least 180 min during constant insulin infusion and increased amino acid delivery to the muscle [44–46].

The stimulation of the muscle protein anabolic response to hyperinsulinaemia–hyperaminoacidaemia by LCn-3PUFA supplementation occurred in the absence of significant changes in whole-body insulin-mediated glucose disposal. This is consistent with the lack of an effect of LCn-3PUFAs on insulin-mediated Akt phosphorylation in muscle, but contradicts the results from studies in animals [2–5] and also some studies in human subjects [53,54]. For example, Delarue et al. [53] demonstrated that 1.8 g of fish oil/day, which is rich in LCn-3PUFAs, given for 3 weeks, diminished the insulin resistance of glucose metabolism as a consequence of dexamethasone treatment in healthy subjects. In addition, Popp-Snijders et al. [54] demonstrated that 3 g of fish oil/day, given for 8 weeks, improved insulin sensitivity in subjects with Type 2 diabetes mellitus. However, most studies in human subjects failed to show a beneficial effect of LCn-3PUFAs on insulin sensitivity (reviewed in detail by Fedor and Kelley [6]). It is possible that the simultaneous administration of glucose and amino acids and the increased mTOR signalling after LCn-3PUFA supplementation in our present study masked a potential beneficial effect of LCn-3PUFAs on glucose metabolism. There is evidence that increased amino-

acid-induced mTOR signalling inhibits insulin sensitivity [55,56] and administration of rapamycin, a known inhibitor of mTOR, increases glucose uptake during a hyperaminoacidaemic–hyperinsulinaemic–euglycaemic clamp in healthy men [57]. Nevertheless, protein/amino acids have a glucose-lowering effect because co-ingestion of glucose and protein/amino acids increases plasma insulin concentration to a greater extent than glucose ingestion alone [58]. It is also possible that, because our subjects were young and healthy and had no signs of insulin resistance, LCn-3PUFA supplementation could not further increase their insulin sensitivity.

We elected to not measure potential changes in muscle mass during the 8 weeks of LCn-3PUFA supplementation in our present study because to do so would have required a much bigger sample size and most probably a longer duration of the intervention; changes in muscle protein metabolism, on the other hand, precede the corresponding changes in muscle mass and therefore ought to occur sooner after the start of the intervention. We also expected the effect of LCn-3PUFAs on muscle protein synthesis to be greater (and thus more easily detectable with a small number of subjects) than their effect on muscle mass because the changes in muscle protein metabolism persist for only a few hours a day in sedentary individuals (during increased amino acid and insulin availability). In fact, with nine subjects we were able to demonstrate significant changes (in the order of ~30%) in the rate of muscle protein synthesis and the concentration of phosphorylated signalling elements in muscle during hyperinsulinaemia–hyperaminoacidaemia. Furthermore, we measured significant increases in both the muscle protein/DNA ratio (an index of muscle cell size [21]) and the muscle protein concentration (per mg of wet weight of muscle) which suggest that LCn-3PUFA may have exerted an overall muscle anabolic effect in the order of 1–2% of muscle mass gain (i.e. the appendicular skeletal muscle mass in our subjects at the beginning of the study was 24 kg, the alkali-soluble protein concentration in muscle was ~11% and increased by ~15%, equivalent to a protein gain of ~0.4 kg). Therefore it is unlikely that we would have obtained meaningful results had we measured FFM or thigh muscle volume in our present study. However, if confirmed in future studies, changes in muscle mass of this magnitude over such a short period of time would certainly be of clinical importance considering that the decline in muscle mass, which starts at about 50 years of age is 0.2–0.5%/year in healthy subjects [59,60] and increased morbidity is demonstrable with as little as a 5% loss of muscle mass [61]. Improvements in muscle mass of this magnitude will also be clinically important in other muscle-wasting conditions, such as cancer cachexia, and there is some evidence in the literature already that LCn-3PUFA supplementation may spare lean body mass in this population [62].

In summary, we have shown that LCn-3PUFA supplementation in healthy 25–45-year-old individuals increases mTOR signalling and the anabolic response of muscle protein synthesis to hyperinsulinaemia-hyperaminoacidaemia, which resulted in increased muscle cell size (protein/DNA ratio) and protein concentration. These results confirm and expand upon our previous findings obtained in older adults [9] and support the notion of a direct muscle protein anabolic effect of LCn-3PUFAs. Furthermore, these results provide a good basis for future research concerning the interaction between muscle protein and lipid metabolism.

AUTHOR CONTRIBUTION

Gordon Smith was involved in conducting the study, processing the study samples, collecting the data, performing the final data analyses, and writing the manuscript. Philip Atherton and Michael Rennie were involved in processing the study samples, collecting the data and writing the manuscript. Dominic Reeds and Selma Mohammed were involved in conducting the study. Debbie Rankin was involved in sample processing and sample analyses. Bettina Mittendorfer was involved in designing and conducting the study, processing the study samples, collecting the data, performing the final data analyses and writing the manuscript.

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■ SUPPLEMENTARY ONLINE DATA

Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia–hyperaminoacidaemia in healthy young and middle-aged men and women

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See the following page for Supplementary Table S1.

Table S1 Plasma phenylalanine, leucine and glucose concentrations, and phenylalanine and glucose TTRs during basal post-absorptive conditions and during the hyperinsulinaemic-hyperaminoacidaemic clampValues are means \pm S.E.M.

Time (min)	Concentration						Enrichment (TTR)			
	Phenylalanine (μ M)		Leucine (μ M)		Glucose (mM)		Phenylalanine		Glucose	
	Before	After	Before	After	Before	After	Before	After	Before	After
Basal conditions										
60	59 \pm 5	56 \pm 4	123 \pm 5	111 \pm 6	5.06 \pm 0.09	5.11 \pm 0.12	0.0921 \pm 0.0045	0.0924 \pm 0.0042	0.0177 \pm 0.0006	0.0178 \pm 0.0005
90	56 \pm 4	55 \pm 5	117 \pm 5	108 \pm 8	—	—	0.0957 \pm 0.0028	0.0923 \pm 0.0032	0.0183 \pm 0.0005	0.0182 \pm 0.0007
120	53 \pm 5	60 \pm 4	108 \pm 6	122 \pm 6	4.93 \pm 0.08	5.12 \pm 0.12	0.1007 \pm 0.0024	0.0963 \pm 0.0033	0.0200 \pm 0.0008	0.0192 \pm 0.0006
150	54 \pm 5	53 \pm 4	109 \pm 7	109 \pm 8	—	—	0.1006 \pm 0.0028	0.1019 \pm 0.0031	0.0200 \pm 0.0006	0.0201 \pm 0.0006
180	52 \pm 7	52 \pm 4	105 \pm 9	103 \pm 6	4.97 \pm 0.08	5.00 \pm 0.10	0.1051 \pm 0.0029	0.1046 \pm 0.0055	0.0239 \pm 0.0008	0.0244 \pm 0.0009
210	59 \pm 6	58 \pm 7	120 \pm 9	123 \pm 11	4.85 \pm 0.07	4.90 \pm 0.12	0.1066 \pm 0.0025	0.1022 \pm 0.0032	0.0240 \pm 0.0007	0.0238 \pm 0.0009
220	—	—	—	—	4.83 \pm 0.09	4.96 \pm 0.11	—	—	0.0240 \pm 0.0007	0.0240 \pm 0.0009
230	—	—	—	—	4.84 \pm 0.09	4.90 \pm 0.12	—	—	0.0238 \pm 0.0007	0.0234 \pm 0.0011
240	56 \pm 5	56 \pm 4	108 \pm 7	117 \pm 7	4.84 \pm 0.10	4.94 \pm 0.13	0.1064 \pm 0.0028	0.1045 \pm 0.0037	0.0235 \pm 0.0007	0.0245 \pm 0.0009
Hyperinsulinaemic-hyperaminoacidaemic clamp										
270	87 \pm 9	94 \pm 9	173 \pm 9	194 \pm 15	5.06 \pm 0.12	5.00 \pm 0.16	0.1014 \pm 0.0044	0.0970 \pm 0.0020	0.0231 \pm 0.0006	0.0240 \pm 0.0007
300	93 \pm 9	96 \pm 9	164 \pm 9	171 \pm 13	5.27 \pm 0.08	5.34 \pm 0.10	0.0966 \pm 0.0025	0.0956 \pm 0.0030	0.0243 \pm 0.0006	0.0249 \pm 0.0006
330	107 \pm 14	97 \pm 8	179 \pm 20	155 \pm 15	5.55 \pm 0.10	5.46 \pm 0.12	0.0931 \pm 0.0019	0.0966 \pm 0.0031	0.0256 \pm 0.0005	0.0265 \pm 0.0005
360	92 \pm 7	109 \pm 7	152 \pm 13	163 \pm 8	5.57 \pm 0.07	5.41 \pm 0.14	0.0944 \pm 0.0033	0.0945 \pm 0.0030	0.0265 \pm 0.0005	0.0270 \pm 0.0005
390	98 \pm 8	101 \pm 6	147 \pm 6	147 \pm 8	5.35 \pm 0.08	5.27 \pm 0.08	0.0976 \pm 0.0028	0.0898 \pm 0.0026	0.0272 \pm 0.0005	0.0276 \pm 0.0005
400	—	—	—	—	5.47 \pm 0.05	5.40 \pm 0.07	—	—	0.0277 \pm 0.0006	0.0280 \pm 0.0005
410	—	—	—	—	5.42 \pm 0.06	5.40 \pm 0.07	—	—	0.0276 \pm 0.0005	0.0276 \pm 0.0004
420	109 \pm 12	104 \pm 7	159 \pm 12	158 \pm 16	5.47 \pm 0.09	5.37 \pm 0.06	0.0986 \pm 0.0023	0.0967 \pm 0.0028	0.0277 \pm 0.0006	0.0275 \pm 0.0005

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