Blunting of insulin inhibition of proteolysis in legs of older subjects may contribute to age-related sarcopenia1–3

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ABSTRACT
Background: Reduced postprandial muscle proteolysis is mainly due to increased insulin availability. Whether rates of proteolysis in response to low physiologic doses of insulin are affected by aging is unknown.

Objectives: We tested the hypothesis that suppression of leg protein breakdown (LPB) by insulin is blunted in older subjects, together with blunted activation of Akt–protein kinase B (PKB).

Design: Groups of 8 young [mean (±SD) age: 24.5 ± 1.8 y] and older (65.0 ± 1.3 y) participants were studied during euglycemic (5 mmol/L), isoaminoacidemic (blood leucine ∼ 120 μmol/L) clamp procedures at plasma insulin concentrations of ∼5 and ∼15 μIU/mL for 1.5 h. Leg amino acid balance, whole-leg protein turnover (as dilution of amino acid tracers), and muscle protein synthesis were measured with d5-phenylalanine and [1,2-13C2]leucine. The kinase activity of muscle Akt-PKB and the extent of phosphorylation of phosphoproteins.

Results: Basal LPB rates were not different between groups (66 ± 11 compared with 51 ± 10 nmol leucine · 100 mL leg−1 · min−1 and 30 ± 5 compared with 24 ± 4 nmol phenylalanine · 100 mL leg−1 · min−1 in young and older groups, respectively). However, although insulin at ∼15 μIU/mL lowered LPB by 47% in the young subjects (P < 0.05) and abolished the negative leg amino acid balance, this caused only a 12% fall (P > 0.05) in the older group. Akt-PKB activity mirrored decreases in LPB. No differences were seen in muscle protein synthesis or associated anabolic signaling phosphoproteins.

Conclusions: At moderate availability, the effect of insulin on LPB is diminished in older human beings, and this effect may be mediated through blunted Akt-PKB activation. Am J Clin Nutr 2009;90:1343–50.

INTRODUCTION
Sarcopenia appears to be an inevitable consequence of aging and affects both sexes and all races. The prevalence of disabling sarcopenia is estimated as between 13% and 24% for men and women aged 65–70 y and is >50% in those aged >80 y (1, 2). This progressive loss of lean tissue occurs as a result of a diurnal net imbalance between the rates of muscle protein breakdown (MPB) and muscle protein synthesis (MPS), but the exact nature of the failure of muscle maintenance is currently unknown.

Insulin promotes muscle accretion by inhibition of proteolysis, apparently in a dose-dependent fashion (3–11). The physiologic inhibitory dose of insulin is less clear. Louard et al (6), Gelfand and Barrett (10), and Pozefsky et al (12) have proposed that maximal inhibition of proteolysis occurs at modest plasma insulin concentrations (∼30 μIU/mL), whereas other investigators have suggested maximal inhibition occurs at concentrations exceeding the physiologic range (>400 μIU/mL), providing that amino acid (AA) availability is maintained at postabsorptive concentrations (9, 11, 13). Two reports from the same laboratory have concluded, against the accepted paradigm, that insulin, infused directly into the femoral artery, does not suppress rates of proteolysis (14, 15), but the data in these studies may be susceptible to a type 1 error because of insufficient participants and high variability.

When the responses of whole-body protein turnover are compared in young and older subjects, it appears that at insulin and AA concentrations elevated above basal, postabsorptive values, rates of protein breakdown are higher in older subjects (16, 17), although this differential appears to be lost at higher insulin concentrations. Measurements of leg balance and leg protein breakdown (LPB), assumed to be due mainly to muscle, are not different in the basal state in young and older subjects (18). However, a possibility exists that, during feeding when insulin secretion is stimulated, there may, in addition to a decreased rate of MPS, also be blunted suppression of MPB. Furthermore, most of the effect of insulin in suppressing LPB appears to have occurred in full by ∼30 μIU/mL and may occur at even lower concentrations (19). Thus, we hypothesized that LPB would be suppressed in the young subjects at insulin availability as low as ∼15 μIU/mL but to a lesser extent in older subjects. [We aimed to achieve a degree of insulinenia comparable with that which follows a low–glycemic index meal such as wholemeal pasta (20, 21).] This hypothesis has never, to our knowledge, been tested directly in a systemic fashion. The aims of this work were 1) to fill this gap and 2) to determine, in

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addition, whether alterations of activation by phosphorylation of components of muscle cell signaling pathways, particularly activation of Akt-protein kinase B (PKB), and short-term changes in gene expression of proteolytic enzymes might provide clues to the mechanisms involved.

SUBJECTS AND METHODS

The protocol was approved by the University of Nottingham Medical School Research Ethics Committee, conducted in accordance with International Conference on Harmonization, World Health Organization Good Clinical Practice standards, and began recruitment in 2007. Written informed consent was obtained from all participants. Two groups of 8 young [age (mean ± SEM); 24.5 ± 1.8 y] and older (65.0 ± 1.3 y) healthy volunteers, 4 men and 4 women per group (Table 1) participated after completion of medical screening, which included measurement of postabsorptive blood glucose and insulin concentrations to exclude individuals with insulin resistance or diabetes as defined as a value of >4.65 with the homeostasis model assessment of insulin resistance (22).

Study protocol

All participants attended the clinical investigation suite at 0800 after a 12-h fast, during which they were permitted water ad libitum. Participants were asked to eat their usual diet and to refrain from strenuous exercise in the preceding 72 h. Body composition data were acquired by dual-energy X-ray absorptiometry (Lunar Prodigy II; General Electric, Amersham, United Kingdom) unless otherwise specified (Figure 1). All reagents used were purchased from Sigma-Aldrich (Poole, England). Muscle samples for RNA extraction were directly frozen. All samples were stored for RNA extraction, were determined by comparison to a standardized sample, with the use of norleucine as an internal standard.

To achieve target plasma insulin concentrations of ≈5 and ≈15 μU/mL, each for 1.5 h consecutively, insulin (Humulin S; Lilly UK, Basingstoke, United Kingdom) was infused at 2 and 10 μU · m² · body surface area⁻¹ · h⁻¹ accompanied by a constant infusion of octreotide (Sandostatin; Novartis UK, Cambridge, United Kingdom; 30 ng · kg⁻¹ · min⁻¹). Glucagon was not given because it has been shown to have no effect on AA metabolism (24). Euglycemic isoaminoacidemic conditions were maintained with the use of 20% dextrose (infused at rates determined by the extent of deviation of arterialized blood glucose from 5 mmol/L) and, for the later part of the study, a mixed AA preparation (25.5 mg · kg⁻¹ · h⁻¹; Glamin; Fresenius Kabi, Bad Homburg, Germany). The stable isotope–labeled AA tracers, [1,2-13C₂]leucine (99% 13C) and D₅-phenylalanine (98% ²H) (both from Cambridge Isotopes Ltd, Cambridge, MA), were administered in primed, constant infusions ([1,2-13C₂]leucine: 0.75 mg/kg prime and 1 mg · kg⁻¹ · h⁻¹; D₅-phenylalanine: 0.3 mg/kg prime and 0.6 mg · kg⁻¹ · h⁻¹). Doppler ultrasound (Nemio 17; Toshiba Medical Systems Ltd, Crawley, United Kingdom) measurements of blood flow through the common femoral artery were taken just distal to the inguinal ligament at frequent intervals. Muscle biopsies from vastus lateralis were obtained under local anesthetic with the use of the conchotome technique (25).

Blood samples were collected at each time point into pre-treated tubes containing lithium heparin and immediately stored on ice before separation at 2000 × g for 20 min; plasma was kept for later analysis at −80°C. AA fluxes were calculated from plasma AA concentrations after correction for hematocrit. Muscle tissue, other than that stored for RNA extraction, was rapidly washed in ice-cold phosphate-buffered saline on collection and blotted before being frozen in liquid nitrogen. Muscle samples for RNA extraction were directly frozen. All reagents used were purchased from Sigma-Aldrich (Poole, United Kingdom) unless otherwise specified (Figure 1).

Insulin, C-reactive protein, cortisol, and free fatty acids

The concentrations of plasma insulin, C-reactive protein, and cortisol were determined by enzyme-linked immunosorbent assays, according to manufacturer instructions (DRG Instruments GmbH, Marburg, Germany). Plasma free fatty acids were measured with the use of a colorimetric method (Roche Applied Science, Indianapolis, IN).

AA analysis

Equal volumes of plasma and 10% sulfosalicylic acid were mixed and cooled at 4°C for 30 min. The samples were spun at 1000 × g to remove the precipitated protein and passed through a 0.22-μm filter before analysis by a dedicated AA analyzer (Biochrom 30; Biochrom, Cambridge, United Kingdom) with the use of a lithium buffer separation. All 20 AA concentrations were determined by comparison to a standardized sample, with the use of norleucine as an internal standard.

Myofibrillar protein isolation

Muscle (30 mg) was minced with the use of fine scissors in ice-cold extraction buffer (0.02 mol Tris/L, 0.15 mol NaCl/L, 0.1 mol EDTA/L, 0.1% Triton X). The homogenate was centrifuged at
1600 × g for 20 min, the supernatant fluid was removed, and the myofibrillar-collagen pellet was resuspended in 0.3 mol NaOH/L. The soluble myofibrillar protein and the insoluble collagen were separated by centrifugation. The myofibrillar fraction was precipitated with 1 mol perchloric acid/L, and the pellet was washed twice with 70% ethanol. Myofibrillar protein was hydrolyzed in 0.05 mol HCl/Dowex 50W-X8-200/L at 110°C overnight (26), and the liberated AAs were purified then eluted/lysed in 0.05 mol HCl/L. Lipids were removed by ethyl acetate extraction, washed twice with 70% ethanol. Myofibrillar protein was hy-
separated by centrifugation. The myofibrillar fraction was pre-
pared. Concentrations and enrichments were determined by gas chromatography combustion isotope ratio mass spectrometry (Delta-plus XL; Thermo Fisher Scientific, Hempstead, United Kingdom) with the use of appropriate internal standards (28).

**Plasma AA labeling**

To determine labeling (atom % excess; APE) and concen-
trations of arterialized venous and venous leucine, phenylalanine, and α-ketoisocaproate (α-KIC), plasma was deprivedmined with 100% ethanol, dried, and, for AA analysis, resuspended in 0.5 mol HCl/L. Lipids were removed by ethyl acetate extraction, and the AAs were converted to their tert-butylidemethylsilyl derivatives. α-KIC was converted to its quinoxalinol derivative and separated by extraction into ethyl acetate, which was then evaporated, and the tert-butylidemethylsilyl derivative was prepared. Concentrations and enrichments were determined by gas chromatography–mass spectrometry (GC-MS) with the use of a Trace DSQ GC-MS (Thermo Fisher Scientific, Hemel Hempstead, United Kingdom) with the use of appropriate internal standards (28).

**Calculations**

The rate of MPS between the biopsies was calculated with the use of standard equations, fractional protein synthesis [in %/h; (ΔEp/Ep × 1/t) × 100 (29)], where ΔEp is the change in labeling of muscle protein leucine between 2 biopsy samples, Ep is the mean enrichment over time of the precursor for protein synthesis (taken as venous α-KIC 13C2 labeling), and t is the time in hours between biopsies. Venous α-KIC was chosen to represent the immediate precursor for protein synthesis, ie, leucyl-r-RNA (30).

Whole-body leucine flux was calculated with the use of the reciprocal pool model, Flux = i × (Ei/Ep) − 1, where i is the rate of tracer infusion (in μmol · kg⁻¹ · h⁻¹), Ei is the enrichment of the infusate, and Ep is the enrichment in the pool chosen to represent the precursor for protein synthesis, ie, venous KIC (31).

Leg protein flux (ie, breakdown) was calculated from the arteriovenous dilution of [13C]phenylalanine and [1,2-13C2]leu-
cine tracers with the following equation: [(Ep/Ea) − 1] × Cα × blood flow (32), where Eα and Ea are the values of AA labeling at steady state in arterial and femoral venous plasma, Cα is the mean concentration in the arterial blood with blood flow in mL/100 mL leg, adjusted for leg volume (assessed by dual-energy X-ray absorptiometry). For each 1.5-h period the values for enrichment and concentration were obtained from the mean of 4 separately analyzed samples, collected over the last hour. The net AA balance was calculated as the difference in arterial and venous concentrations multiplied by the plasma flux, which was also the average of 4 blood flow readings taken immediately before each of the blood samples and adjusted with each subject’s hematocrit.

**Western analysis of signaling proteins**

Phosphorylated protein concentrations of eukaryotic elonga-
tion factor 2 (eEF2Thr56), p70 ribosomal S6 kinase (Thr389), eu-
karyotic initiation factor 4 binding protein 1 Thr37/46, and Akt PKB kinase activity (through phosphorylation of pseudosubstrate glycogen synthase kinase 3βSer202/204) were determined by Western blotting. Proteins were extracted from ≈30 mg of crudely minced muscle in ice-cold buffer (10 μL/mg muscle) containing 50 mmol Tris HCl/L, 0.1% Triton X-100, 1 mmol EDTA/L, 1 mmol EGTA/L, 0.1% 2-mercaptoethanol, 10 mmol β-glycerophosphate/L, 0.5 mmol sodium orthovanadate/L, and complete protease inhibitor cocktail (Roche, West Sussex, United Kingdom). A Bradford assay was used to quantify protein concentrations of 10,000 g supernatant fluids. For PKB kinase activity, Akt-PKB was im-
munoprecipitated from 300 μg protein before incubation with 1 mmol ATP/L and glycogen synthase kinase 3βSer202/204 were phosphorylated (taken as venous α-KIC 13C2 labeling), and for kinase reactions) was loaded onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Criterion XT Bis-Tris; Bio-Rad, Hemel Hempstead, United Kingdom) for electrophoresis at 150 V for ≈75 min and electroblotted to polyvinylidene difluoride membranes (Bio-Rad). After incubation for 1 h with 5% bovine serum albumin in TBS-T (Tris-buffered saline and 0.1% Tween-20), membranes were incubated overnight with primary antibody against the aforementioned tar-
gets at 4°C (New England Biolabs). Membranes were then washed with TBS-T and incubated for 1 h at room temperature with the horseradish peroxidase–conjugated antirabbit secondary
antibody (New England Biolabs), before further washing with TBS-T and incubation for 5 min with enhanced chemiluminescence reagents (enhanced chemiluminescence kit; Immunostar; Bio-Rad). Blots were imaged and quantified with the use of the Chemidoc XRS system (Bio-Rad). Immunoblots were not repeated because the laboratory CV between sample-to-sample ratios on repeated gels is low (~10%).

**Gene expression**

Total RNA was extracted from ~20 mg of each muscle sample with 0.5 mL Tri-reagent as per the manufacturer’s protocol. Integrity and quantity of RNA was assessed by gel electrophoresis and luminescence. QuantiTect reverse transcription kit (Qiagen Ltd, Crawley, United Kingdom) was used for cDNA synthesis and genomic DNA elimination. Real-time polymerase chain reaction was performed with SYBR Green supermix (Bio-Rad) –3

Power calculation and statistical analyses

The sample size was determined prospectively with a power calculation and taking a population variance of 15% (based on previous laboratory data) and CV of laboratory techniques also of 15%, to detect (with 80% confidence at the 5% significance level) change in the primary endpoint, ie, a difference between rates of LPB between the 2 age groups at each insulin concentration. A post hoc sample size calculation was performed with the actual data. Given the large effect of insulin, and that the groups were of different size with 14 df and with variance set for both groups at 15%, to detect (with 80% confidence at the 5% significance level) a change in the primary endpoint, ie, a difference between rates of LPB was substantially suppressed in young participants (each ~5 μIU/mL, P < 0.02, in young compared with older groups, respectively).

The phenylalanine balance differed with insulin availability significantly in the young group but not the old group (Figure 2). In the young group, at 5 μIU/mL insulin balance was also significantly different from zero (or neutral balance), but at 15 μIU/mL insulin there was no difference from neutral phenylalanine balance. The older group did not differ from neutral balance at either insulin availability. Whole-body leucine flux did not differ between participants of different ages or when studied under different insulin availability, with values of 130.3 ± 7.7 and 133.4 ± 9.7 μmol · kg⁻¹ · h⁻¹ for young participants and 122.6 ± 10.4 and 120.8 ± 11.5 μmol · kg⁻¹ · h⁻¹ for older participants (each ~5 and ~15 μIU/mL, respectively).

LPB, whether determined by leucine or phenylalanine tracer dilution, did not differ between the groups at insulin concentrations of ~5 μIU/mL (Figure 3). At the higher insulin concentration, LPB was substantially suppressed in young participants (59% with phenylalanine and 47% with leucine tracer; both P < 0.05), whereas LPB was reduced in the older group to a lesser extent, achieving significance when calculated with phenylalanine but not leucine (23% and 12% reduction from basal values, respectively).

No differences were observed between groups in the rates of MPS as determined by the incorporation of [1,2-13C₂]leucine, and they did not change with increased insulin availability (fractional protein synthesis of quadriceps muscle in young compared with...
older participants: $0.051 \pm 0.005$ compared with $0.043 \pm 0.005\%/h$ at $\approx 5$ μIU/mL and $0.046 \pm 0.005$ compared with $0.048 \pm 0.005\%/h$ at $\approx 15$ μIU/mL$^2$). The values for leg protein synthesis showed a similar pattern (data not shown).

In both young and older subjects, Akt-PKB activity rose with the increase of insulin to $\approx 15$ μIU/mL (Figure 4). Phosphorylation status of eEF2, p70 ribosomal S6 kinase, eukaryotic initiation factor 4 binding protein 1, and FoxO1a did not change in either group at either insulin concentration (Figure 5). The gene expression of the ubiquitin ligases MuRF1 and MAFBx, the C2 proteosomal subunit, cathepsin L, and ubiquitin also remained unchanged (Figure 6).

**DISCUSSION**

To our knowledge this is the first study reporting a comparison of the effects of low physiologic amounts of insulin on the rate of leg proteolysis at postabsorptive AA concentrations in young and older human beings. It appears that aging interferes with the normal suppression effect of LPB in response to a moderate rise in plasma insulin concentration, despite there being no differences in the basal state in leg (probably mainly muscle) proteolysis.

These findings cannot simply be explained by differences in body composition or conventionally described insulin sensitivity in respect of glucose metabolism because in all respects the young and older healthy volunteers were well matched. The older participants had no evidence of pathologic sarcopenia, and all participants had normal calculated values for insulin sensitivity, ruling out the possibility of incipient diabetes contributing to impaired muscle metabolism. Furthermore, availability of free fatty acids did not differ at any time point. Blood flow was significantly lower in the older participants, which is not a novel finding, and may be explained by the phenomenon of endothelium-dependent vasodilator blunting in response to insulin with aging (14, 34).

The reported dose-dependent effect of insulin on blood flow to the limb (7, 15, 35) occurs at higher insulin concentrations than present during the second period of this study. Thus, it is likely that the delivery of insulin was too low to elicit an effect on blood flow. Indeed, no change in bulk femoral artery blood flow with small changes to insulin concentrations has been reported previously (15).

Other researchers have measured whole-body leucine flux under similar conditions and have shown that protein breakdown is suppressed when plasma insulin is increased to absorptive concentrations ($\leq 30$ μIU/mL) with the degree of suppression being reduced in older subjects (16, 17). However, the whole-body data we present here did not confirm those findings; we found no differences in whole-body leucine flux with a moderate increase in insulin availability or between participants of different ages.

The differences in these findings from those of the French workers may be accounted for by differences in body composition. The body composition of participants, particularly young participants, differed markedly between studies. Mean values for body mass index and proportion of fat mass were substantially lower in the subjects studied by Boirie et al (17) than in our volunteers, so that the relative contribution of muscle (rather than splanchnic protein turnover) to whole-body flux would be greater. The converse would hold for less lean subjects. Consequently, suppression of MPB by insulin might be offset by enhanced and
insulin-resistant splanchnic proteolysis with whole-body net balance possibly remaining unchanged. This differential effect of insulin on splanchnic and muscle tissue has been described previously, albeit not under isoaminoacidemic conditions (36, 37).

Most previous estimates of MPB in the fasted postabsorptive state do not differ with age (14, 18, 38–40), in accordance with data presented here for both flux of unlabeled phenylalanine and dilution of stable isotope-labeled AA across the leg. In young adults, suppression of MPB appears dependent on the dose of insulin, during isoaminoacidemia (3–11). Thus, postabsorptive plasma AA concentrations were maintained throughout both phases of the study with the use of a proprietary AA preparation, such that arterial EAA concentrations crept up slightly above basal values for both age groups toward the end of the study, albeit to the same degree. Nevertheless, the rise in EAA availability was not sufficient to stimulate MPS or anabolic signaling protein phosphorylation, both of which remained at postabsorptive values for both phases of the study. Furthermore, age did not affect rates of basal mixed MPS, in keeping with other published results (18, 39, 41–43). We and others have previously shown that, regardless of AA concentrations, insulin has no effect on MPS, beyond that of altering AA delivery to the tissues (3, 4, 10, 19), and this is also confirmed by the current results.

As mentioned earlier, reports suggest that insulin can be infused directly into the femoral artery without eliciting a change in the rate of local MPB (14, 15). The investigators report that venous AA concentrations are maintained within 10% of basal when local infusion of insulin is used, although they also report that phenylalanine concentration within the tissue falls significantly in both young and older participants, especially in the older group (reduction 25%). Furthermore, Fujita et al (15) did actually report falls in the rate of appearance of phenylalanine at both low (224%) and high (235%) insulin concentrations, albeit only reaching significance at the higher concentration. We have no explanation for their results except the existence of a possible type 1 error because of insufficient numbers of subjects given the variability experienced.

Rasmussen et al (14) compared responses to local insulin infusions between young and older healthy volunteers, although insulin concentrations within the femoral vein were doubled in the older group (young group: 40.3 ± 1.0 μIU/mL; older group: 83.4 ± 6.2 μIU/mL; P < 0.05). When results from young volunteers with comparably high insulin concentrations are compared with the older group data, there is a clear trend toward inhibition of proteolysis.

To summarize, in our study we maintained plasma leucine concentrations well and found that MPB was markedly suppressed in young subjects with moderately increased insulin availability infusion in accord with most of the published literature, irrespective of systemic or close arterial infusion of insulin, including that by Gelfand et al (10), who also used close arterial infusion of insulin.
In terms of signaling proteins, Akt-PKB kinase activity (not simply phosphorylation status), which is frequently used as a proxy of “insulin action” (44), rose with increased insulin availability, as expected. However, in the present study increased Akt-PKB activity had no effect on FoxO1α phosphorylation status, and this coupled to the failure to detect down-regulation of “atrogene” (i.e., MAFBx, MuRF1; Figure 6) mRNA expression (45) reduces the likelihood of inhibition of MPB by the Akt-PKB-FoxO1α-atrogene axis.

Both the anabolic sensing-signaling protein mammalian target of rapamycin (mTOR) and the elongation factor eEF2 appear, at least in vitro, to be activated by insulin (46) and are strongly implicated in the regulation of protein translation. However, our data show that at low physiologic insulin concentrations, despite Akt-PKB signaling, mTOR and eEF2 signaling are unaffected, which may explain the lack of protein synthetic response to insulin we report.

What is the broader significance of our work? In real terms, the degree of insulinemia achieved during the second period of this study (≈15 μIU/mL) is comparable with that expected after a “healthy” low–glycemic index meal, such as breakfast or a light lunch, yet it would appear from these results that the normal suppression of proteolysis by insulin at this concentration is impaired with aging. Whether this blunted sensitivity to insulin is also present at higher insulin concentrations, as may occur with meals containing refined carbohydrates, remains to be investigated. Nevertheless, it appears that loss of muscle with aging may be accelerated not only by a blunted anabolic response to AAs but also relative insensitivity to the anti-proteolytic effects of insulin.

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The authors’ responsibilities were as follows—EAW: conducted all clinical studies, obtained tissue and blood samples, analyzed data, and drafted the manuscript; ALS, PJA, RP, DR, and KS: assisted in collection, processing and analysis of tissue and blood samples; and EAW, KS, PJA, and MJR: designed the study, which was based on an idea by MJR; interpreted the data; and critically revised the manuscript. None of the authors declared a conflict of interest.

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![FIGURE 6. Mean (±SEM) fold change in expression of Muscle Atrophy F Box (MAFBx), Muscle RING Finger 1 (MuRF1), ubiquitin, C2 proteosomal subunit, and cathepsin L mRNA in young (open bars) and older (hatched bars) healthy volunteers after 90-min pancreatic clamping at insulin concentrations of ≈15 μIU/mL and ≈5 μIU/mL; n = 8 for both groups. All P > 0.05 for young compared with older volunteers (Student’s t test).](https://academic.oup.com/ajcn/article-abstract/90/5/1343/4598139)


