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ORIGINAL CONTRIBUTION

Livio Luzi
Mauro Giordano
Marianna Caloni
Pietro Castellino

Effects of insulin and amino acids on leucine metabolism in young and middle-aged humans

Summary *Background* Aging is characterized by loss of muscle mass. In healthy subjects this process is associated with hormone and nutritional changes which take place over many decades. *Aim of the study* To investigate the effects of insulin and amino acids on amino acid metabolism in middle-aged humans. *Methods* We evaluated leucine kinetics by means of the intravenous infusion of [1-^{14}C]leucine, in 8 young (age 24±2 yr, BMI 21±2 kg/m^2) and in 6 middle-aged (age 53±4 yr, BMI 26±1 kg/m^2) healthy subjects. Studies were performed under fasting conditions (basal), and after 180 min of euglycemic hyperinsulinemic clamp (study I), or 180 min of euglycemic hyperinsulinemia in combination with an intravenous amino acid infusion (study II). *Results* In the basal state endogenous leucine flux (ELF, an index of proteolysis), normalized for IBW, averaged 1.71±0.12 and 1.66±0.14 μmol/kg · min in young and middle-aged subjects, respectively. Basal leucine oxidation (0.22±0.03 vs 0.28±0.03 μmol/kg · min, $p < 0.05$) was lower in middle-aged with respect to young subjects. Non-oxidative leucine disposal (NOLD, an index of protein synthesis: 1.44±0.11 vs 1.43±0.11 μmol/kg · min) was similar in young and middle-aged subjects, respectively. In response to insulin (study I) the absolute and percent decline of ELF and LOX were similar in young and middle-aged subjects: ELF declined to 1.05±0.06 μmol/kg · min (–39±5 %) and 1.07±0.14 μmol/kg · min (–36±4 %), in young and middle-aged, respectively (both $p < 0.01$ vs basal); LOX declined to 0.21±0.02 μmol/kg · min (–35±3 %), and 0.18±0.05 μmol/kg · min (–28±3 %, $p < 0.05$ vs basal) in young and middle-aged individuals respectively (both $p < 0.01$ vs basal). In contrast, insulin-mediated whole-body glucose uptake was lower in middle-aged subjects (6.6±1.4 mg/kg · min) with respect to young individuals (8.1±1.7 mg/kg · min, $p < 0.05$). During study II (insulin plus AA) a significant rise in NOLD was obtained in both young (1.72±0.10 μmol/kg · min, $p < 0.01$ vs basal) and middle-aged subjects (1.76±0.25 μmol/kg · min, $p < 0.01$ vs basal). Similarly, net leucine balance rose significantly in both young (+0.62±0.13 vs –0.25±0.02 μmol/kg · min, $p < 0.01$ vs basal) and middle-aged subjects (+0.37±0.08 vs –0.22±0.03 μmol/kg · min, $p < 0.01$ vs basal) suggesting that the anabolic response to amino acids is preserved in middle-aged subjects. *Conclusions* In middle-aged subjects we observed 1) a moderate decline in basal leucine oxidation; 2) a normal antiproteolytic response to insulin and a reduction in glucose uptake; and 3) a normal anabolic response to AA plus insulin. In conclusion, the data provide evidence for a normal regulation of protein anabolism and an early dissociation between the metabolic effects of insulin on glucose uptake and proteolysis in middle-aged subjects.

Key words Aging – Glucose Metabolism – Protein Metabolism – Leucine kinetics – Insulin action

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Dr. L. Luzi (✉) · M. Caloni
Università degli Studi di Milano
San Raffaele Scientific Institute
Via Olgettina 60
20132 Milano, Italy
Tel./Fax: 00 39-02-26 43-37 38
E-Mail: luzi.livio@hsr.it

M. Giordano · P. Castellino
Università di Catania, Italy
Istituto di Clinica Medica Generale e Terapia Medica "L. Condorelli"

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Introduction

One of the most prominent features of aging is a progressive decline in the body storage of amino acid and protein, which affects primarily muscular mass and could contribute to decreased strength and physical performance as well as to increased susceptibility to sepsis and trauma [1].

In previous studies it has been demonstrated by us [2] as well as by others [3] that insulin plays a pivotal role in the regulation of protein homeostasis. The major *in vivo* effect of insulin is a dose-dependent decline in proteolysis with an attendant decline in protein synthesis [4]. It has been demonstrated that insulin-induced hypoaminoacidemia may represent a limiting factor for protein synthesis. In fact, when insulin is provided with amino acids, both a stimulation of protein synthesis and an inhibition of proteolysis are observed [2]. Furthermore, it has been demonstrated that amino acid availability is an essential condition in order to achieve a net stimulation of protein synthesis [2, 5].

From a theoretical stand-point, protein malnutrition may derive from either a defective antiproteolytic action of insulin, as occurs in diabetes mellitus [6], or from an impaired response to hyperaminoacidemia, as observed in chronic renal failure and metabolic acidosis [7, 8].

Only a few studies have addressed these issues in aging humans [9] and no data are available in middle-aged and otherwise healthy individuals. In the present study we employed the euglycemic insulin clamp technique in combination with [1-^{14}C] leucine infusion and indirect calorimetry to examine the effect of insulin and amino acid availability on glucose and amino acid metabolism in healthy middle-aged individuals.

Methods

Subject population

Eight healthy young volunteers (4M/4F) and 6 healthy middle-aged subjects (4M/2F) participated in the study protocol. All subjects were within 20% of their ideal body weight (young 104±4%, middle-aged 113±4%) based on the medium frame of the Metropolitan Life Insurance Table (1983). Their BMI were young 21±1 kg/m^2 and middle-aged 26±1 kg/m^2. Table 1 represents BMIs of each middle-aged subject. The mean age was 24±2 and 53±4 years for young and middle-aged adults, respectively. No subject had any evidence of endocrine or other major organ system disease. Liver function tests were normal in all subjects. For at least three days prior to the study all subjects consumed a weight-maintaining diet providing at least 250 g of carbohydrate and between 50–80 g of protein. The purpose and potential risks of the study were explained to all subjects and their voluntary written consent was obtained before their participation. The experimental protocol was approved by the Institutional Ethical Committee.

Table 1 The individual BMI of the middle-aged patients

Pts.	Ht (cm)	Wt (kg)	BMI (kg/m^2)
1	178	80	25.2
2	155	64	26.7
3	174	79	26.2
4	170	90	31.1
5	165	65	23.9
6	163	62	23.3

Experimental protocol

All tests were performed in the post-absorptive state beginning at 08.00 h after a 12 h overnight fast. Healthy volunteers participated in two experimental protocols that were performed in random order at 10–15 day intervals. In each study protocol, a small polyethylene catheter was inserted into an antecubital vein for the infusion of all test substances. A second catheter was placed retrogradely into a wrist vein for blood sampling. The hand was kept in a heated box at 70 °C to ensure arterialization of the venous blood.

At 08.00, a prime (16–22 μCi bolus) continuous (0.20–0.25 μCi/min) infusion of [1-^{14}C] leucine (New England Nuclear, Boston, MA) was begun and continued until the end of the study in combination with a priming dose of bicarbonate (4 μCi). After two hours of isotope equilibration, samples were drawn every 10–15 min from 120 to 180 min for the determination of baseline leucine and alpha-ketoisocaproate specific activities and plasma hormone and substrate determinations. Continuous indirect calorimetry was started after 120 min. Expired air samples were collected at 15 min intervals and bubbled through a CO_2 trapping solution (Hyamine hydroxide: absolute ethanol: 0.1% phenolphthalein 3:5:1). The solution was titrated to trap 1 mmol CO_2/3 ml of the solution. The $^{14}CO_2$ radioactivity was subsequently determined using a Packard Tricarb Scintillation Counter (Packard Instruments, Dower Grove, IL). At the end of the basal period, one of the following study protocols was performed.

Study one – insulin clamp

After 180 minute equilibration period, a prime-continuous infusion of regular insulin (Eli Lilly Co., Indianapolis, IN) was administered at a rate of 40 mU/m^2 · min to acutely achieve and maintain an increment in plasma insulin concentration of approximately 80 μU/ml. The

plasma glucose concentration was maintained at the basal level by determination of the plasma glucose concentration at 5 min intervals and periodically adjusting the infusion rate of a 20% glucose solution to maintain euglycemia [10].

Study two – insulin clamp plus amino acid infusion

The insulin clamp was performed as described above with one exception. A balanced amino acid solution (10% Travasol without electrolyte, Travenol Laboratories, Deerfield IL) was begun at a rate of 0.011 ml/kg · min at the start of the insulin infusion and continued throughout the study as previously described [2].

Respiratory exchange measurements

In all studies, respiratory exchange measurements were performed as previously described [11]. Briefly, a plastic ventilated hood was placed over the head of the subject and made airtight around the neck. A slight negative pressure was maintained in the canopy to avoid loss of the expired air. The carbon dioxide and oxygen content of the expired air were continuously measured by a Deltatrac Metabolic Monitor (Sensormedics, Anaheim, CA).

Analytical determinations

Plasma leucine and α-KIC specific activities were measured as previously described [12]. Plasma leucine concentration was determined using an amino acid analyzer (System 6300, Beckman, Anaheim, CA). To precipitate plasma proteins, 2.5 ml of 10% sulphosalycilic acid was added to 2.5 ml of plasma, and a 1 ml aliquot of the supernatant was analyzed in duplicate for plasma amino acid concentration. One milliliter of the remaining supernatant was placed in duplicate on a Dowex 50 G cation exchange resin column (Bio-Rad Laboratories, Richmond, CA) and the free amino acid fraction was eluted with 4 N NH_4OH, subsequently dehydrated, and reconstituted in water. Scintillation fluid (10 ml) was added to each vial and ^{14}C radioactivity was measured in a Packard Tricarb Scintillation Counter (Packard Instruments, Dower Grove, IL). Plasma α-KIC specific activity was measured using a modification of the method previously described by Nissen et al. [12]. Plasma (1 ml) was placed in duplicate on a Dowex 50 G cation exchange resin column (Bio-Rad Laboratories), and the free α-ketoacid fraction was eluted with 4 ml of 0.01 N HCL in 50-ml culture tubes. Methylene chloride (35 ml) was added, and after shaking vigorously for 1 min the tube was centrifuged for 5 min at 2,000 rpm to extract the free α-ketoacid fraction from plasma. After decantation of the supernatant, the α-ketoacid was extracted in 350 µl of 0.2 M NaH_2PO_4 at pH 7. After a brief centrifugation 200 µl of the supernatant was injected into a high-performance liquid chromatographic system. The system utilizes a C_{18} reverse-phase column (Waters Nova-Pak, 0.3×30 cm) that was eluted with 2% acetonitrile in 0.1 NaH_2PO_4 buffer (pH 7.0) at a rate of 1.4 ml/min. Absorbance of KIC was monitored at 206 nm. Radioactivity eluting with the KIC peak was measured by scintillation counting. The inter-assay and intra-assay variations for the determination of [^{14}C]leucine specific activity were 4±2 and 5±2%, respectively. More than 98% of the radioactivity collected in the amino acid fraction was in the leucine peak after separation by ion exchange chromatography. The inter-assay and intra-assay variations for the determination of [^{14}C] KIC specific activity were 5±2 and 5±3%. The recovery of [^{14}C] KIC was 68±4%. Plasma insulin and glucagon concentrations were measured with standard radioimmunoassay techniques. Plasma glucose concentration was determined by the glucose oxidase method.

Calculations

Protein metabolism

Whole body leucine flux was calculated with a stochastic model for protein metabolism. The analysis assumes near steady-state conditions. The validity and assumptions of the model have been previously discussed in detail by Golden and Waterlow [13]. Briefly, the model generates the following equations in which total leucine turnover or flux equals Q=S+C=B+I, where S is the total rate of leucine incorporation into protein (or non-oxidative leucine disposal), C is the rate of leucine oxidation, B is the rate of leucine release from protein (endogenous leucine appearance), and I is the rate of exogenous leucine input.

The rate of leucine turnover (Q) is calculated as follows: Q = F/*Leu sp act*, where F is the infusion rate of [^{14}C]leucine [in disintegrations/min (dpm)] and *Leu sp act* is the specific radioactivity of leucine in the plasma compartment under steady-state conditions. The leucine oxidation rate is calculated as follows: C = O/(K · *Leu sp act*), where O is the rate of appearance of $^{14}CO_2$ in the expired air (dpm/min) and K is a correction factor (0.81) that takes into account the incomplete recovery of labeled $^{14}CO_2$ from the bicarbonate pool. An estimate of the rate of leucine incorporation into protein (S) can be calculated as follows: S = Q – C. An estimate of the rate of leucine release into the plasma space from endogenous protein (B) can be calculated as follows: B = Q – I. When subjects are in the post-absorptive state, leucine intake (I) equals zero and B = Q. During the

amino acid infusion, I equals the rate of leucine administration. To calculate rates of leucine turnover and oxidation, we have employed the plasma α-KIC specific activity because it has been suggested that the plasma α-KIC specific activity, the transaminated product of leucine, may provide a better estimate of the specific activity in the intracellular mixing pool [14].

Glucose metabolism

During the insulin clamp studies, the glucose infusion rate was calculated at 20 min intervals and a space correction was applied for over or under filling of the glucose space when appropriate [10]. For data presentation, the mean of the three 20 min intervals from 120 to 180 min is given. In middle-aged subjects, body fat content may be increased.

To account for differences in body composition between young individuals and middle-age subjects, data were normalized for ideal body weight (IBW) as previously described [8] according to the formula: (Flux · IBW)/(BW) · 100.

Statistical analysis

All values are expressed as means ± SE. Comparisons between the basal and the infusion periods were performed using the Student's "t" test for paired data. Intergroup analysis was performed by one-way analysis of variance.

Results

Endogenous leucine flux (Figs. 1 and 2)

In young subjects, normalized endogenous leucine flux (ELF), as estimated from plasma α-KIC specific activity, was similar in studies I and II and averaged 1.70±0.14 μmol/kg · min. Under condition of euglycemic hyperinsulinemia ELF declined by 39±5% to 1.05±0.06 μmol/kg · min ($p < 0.01$ vs basal). In study II, ELF decreased to 1.35±0.13 μmol/kg · min ($p < 0.01$ vs basal).

In middle-aged subjects ELF was similar in studies I and II and averaged 1.67±0.15 μmol/kg · min. In study I, the insulin infusion determined a 36±4% decline in ELF (p=ns vs youth) to 1.07±0.14 μmol/kg · min ($p < 0.01$ vs basal). During amino acid and insulin infusion, study II, ELF was reduced by 30±4% to 1.14±0.11 μmol/kg · min ($p < 0.01$ vs basal).

Leucine oxidation and non-oxidative leucine disposal (Figs. 1 and 2)

In younger subjects, basal leucine oxidation (LOX) and non-oxidative leucine disposal (NOLD) were similar in studies I and II and averaged 0.28±0.03 and 1.43±0.13 μmol/kg · min, respectively. In study I, both LOX and NOLD declined significantly in response to insulin (0.21±0.02 and 0.83±0.05 μmol/kg · min) (both $p < 0.01$ vs basal). In study II during combined amino acid

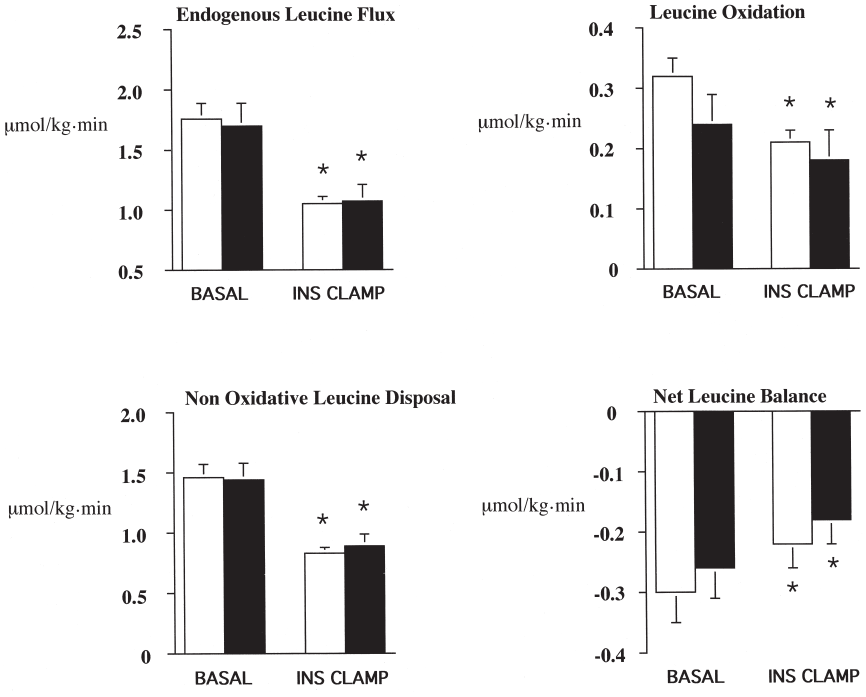

Fig. 1 Endogenous leucine flux (top left), leucine oxidation (top right), non-oxidative leucine disposal (bottom left) and net leucine balance (bottom right) in young (open bars) and middle-aged subjects (full bars) during the basal period (BASAL) and the last hour of the insulin clamp (INS CLAMP). Values are mean ± SE; * $p < 0.05$ vs basal.

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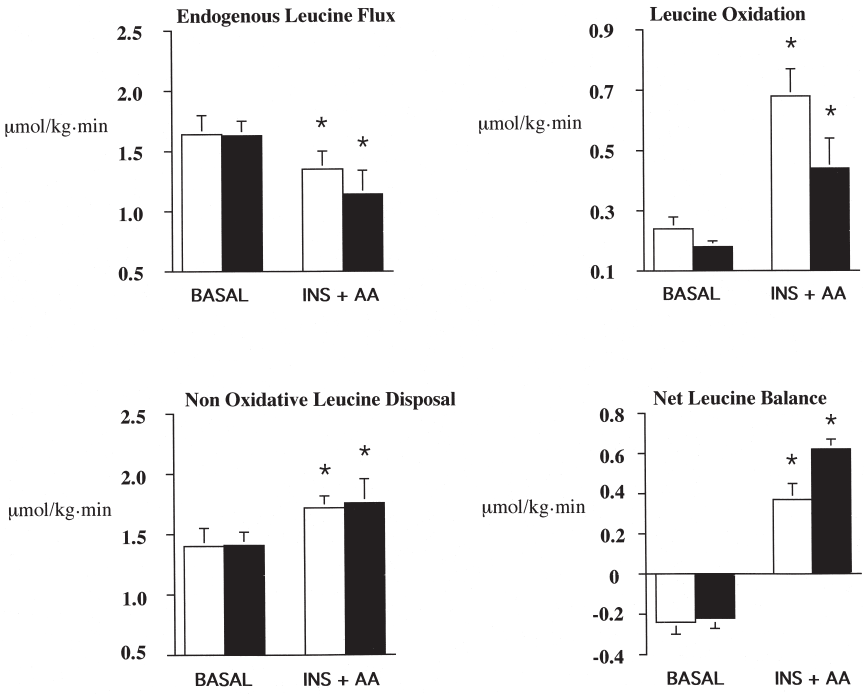

Fig. 2 Endogenous leucine flux (top left), leucine oxidation (top right), non-oxidative leucine disposal (bottom left) and net leucine balance (bottom right) in young (open bars) and middle-aged subjects (full bars) during the basal period (BASAL) and the last hour of the insulin clamp plus amino acid study (INS + AA). Values are mean ± SE; * $p < 0.05$ vs basal.

and insulin infusion, LOX averaged 0.68±0.08 μmol/kg·min ($p < 0.01$ vs basal) and NOLD was 1.72±0.08 μmol/kg·min ($p < 0.01$ vs study I).

In middle-aged subjects, basal LOX and NOLD were similar in studies I and II and averaged 0.22±0.03 and 1.43±0.11 μmol/kg·min, respectively. In response to insulin (study I) LOX (0.18±0.02 μmol·kg·min) and NOLD (0.89±0.10 μmol/kg/min) declined significantly (both $p < 0.01$ vs basal). In study II, LOX and NOLD went to 0.44±0.10 and 1.76±0.25 μmol/kg·min, respectively (both $p < 0.01$ vs study I).

Net leucine balance (Figs. 1 and 2)

The balance between NOLD and ELF represents the net flux of leucine into protein and it provides an index of protein anabolism. In the post-absorptive state, the net leucine balance was negative in both young and middle-aged subjects and averaged –0.25±0.02 and –0.22±0.02 μmol/kg·min. In response to insulin, the leucine balance became less negative in both groups.

In study II the net balance of leucine into protein became positive in both groups and averaged 0.37±0.08 and 0.62±0.13 μmol/kg·min in young and middle-aged subjects, respectively (both $p < 0.01$ vs basal).

Glucose Metabolism (Fig. 3)

In young individuals the rate of glucose infusion (M) required to maintain euglycemia during the last hour of euglycemic insulin clamp averaged 7.8±1.6 mg/kg·min (the value normalized for IBW was 8.1±1.7 mg/kg·min) and was significantly higher than that of middle-age subjects, which averaged 5.8±1.2 mg/kg·min (the value normalized for IBW was 6.6±1.4) ($p < 0.05$ young vs middle-age).

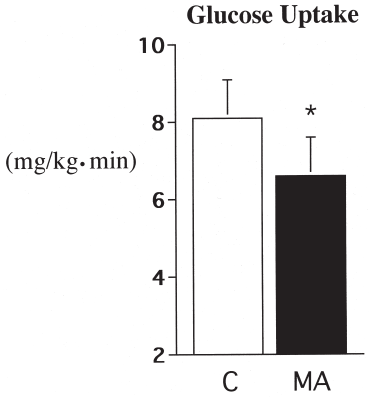

Fig. 3 Whole-body insulin-stimulated glucose metabolism in young (C) (open bars) and middle-aged subjects (MA) (full bars) during the last hour of the insulin clamp (study I).Values are mean ± SE; * $p < 0.05$ vs basal.

Discussion

In the present study we investigated the effects of the administration of insulin alone (or in combination with a mixed amino acid solution) on protein metabolism in middle-aged individuals comparing it with that of young controls. The data show that the ability of insulin to inhibit protein breakdown and the combined effect of insulin and hyperaminoacidemia to stimulate protein synthesis and whole-body protein anabolism are normal in middle-aged individuals. In contrast, the rate of insulin-mediated glucose metabolism is reduced by 20 % in comparison to young controls. This provides evidence for an early dissociation of the effect of aging on insulin action on glucose and protein metabolism.

In the post-absorptive state, whole-body leucine flux as well as leucine oxidation are slightly reduced when expressed for kilogram of body weight in young and middle-aged individuals. In middle-aged individuals, body fat content tends to increase and comparison of body composition of young *vs* old individuals by bioelectrical impedance is not fully validated [16]. To account for differences in body composition, we normalized the data for the ideal body weight, as previously described [8]. With this type of analysis, the present data are in agreement with previous observations and show no evidence of an altered post-absorptive protein turnover or catabolism in aging individuals [9].

However, maintenance of long-term protein balance is the result of an equilibrium between the catabolic state of fasting and the anabolic response to protein feeding [9]. After feeding, the major regulator of whole-body protein balance is the insulin secretory response induced by carbohydrate ingestion and amino acid availability. Previous studies have shown that insulin "per se" is a potent inhibitor of endogenous proteolysis which is reduced by hyperinsulinemia in a dose-dependent fashion [2]. In contrast, oral protein ingestion or intravenous administration of amino acid solution increase amino acid availability and stimulate the rate of their incorporation into body protein [2, 5]. Giordano et al. [17] demonstrated that the stimulation of protein synthesis and net protein balance are a function of the level of peripheral hyperaminoacidemia in a dose-dependent fashion. In the present study middle-aged individuals showed a normal response to the antiproteolytic effect of insulin and to the anabolic action of hyperaminoacidemia. It is of interest that in elderly subjects Boire et al. [18] observed that splanchnic extraction of orally ingested leucine was double that in young controls with no stimulation of protein synthesis. The available data would suggest that the reduction in muscle mass of the elderly can be ascribed to a reduced peripheral availability of ingested amino acid rather than to an intrinsic alteration of the proteolytic/synthetic pathways of the aging muscle. Previous data in aging rats would also support the concept that a lower systemic availability of amino acid during feeding may limit the stimulation of protein synthesis [19].

An additional interest of the present work is that despite a similar insulin-induced inhibition of proteolysis, the rate of insulin-mediated total glucose disposal is significantly reduced. This different effect of age on glucose and amino acid metabolism which is also observed in chronic renal failure [8] and diabetes mellitus [20] may be due to the different site of insulin action on glucose uptake and proteolysis. Muscle has been shown to be the major site of glucose disposal during an insulin clamp [20]. In contrast, both the splanchnic bed and peripheral tissues are responsible of the changes in amino acid exchange during hyperinsulinemia. Alternatively, the data are also consistent with an isolated post-binding defect of insulin action which does not involve the insulin sensitive pathways of endogenous proteolysis [21–25]. In a previous *in vitro* study, we demonstrated a bidirectional modulation of the insulin signaling cascade by amino acids in rat hepatocytes and myocytes [21]. Mainly branched-chain amino acids inhibit the phosphorylation of PI-3-kinase [21], which was linked to glucose uptake [22], and activate the phosphorylation of the p70 S6 kinase, which triggers the protein synthetic process [23]. Therefore, amino acids may act independently from insulin in activating or inhibiting the insulin signal transduction in humans.

A possible misleading factor of the present study is the slightly higher BMI of older subjects. Although this difference should be taken into consideration in the interpretation of our data, it cannot affect the major conclusions of our work, for the following reasons: 1) although statistically significant, the difference is minimal, and the BMI of older patients ranged between 23.3 and 31.1 with only one patient classified as overweight according to standard clinical criteria (BMI > 27 kg/m^2, (24); 2) in a previous study we performed a similar protocol comparing lean and obese subjects (25, 26). Our previous results indicate that obesity per se induces a combined defect of insulin action on leucine and protein metabolism. Clearly the pattern found in the middle-aged patients included in this study is not typical of an effect of obesity per se; 3) a moderate increase of fat mass of the older group would have been present also in the condition of two study groups perfectly matched for BMI. Since the percent of increment of fat mass might have been consistently different in individual patients, the assessment of body composition by means of impedentiometry would have been of little help. Furthermore a full validation of bioelectrical impedence analysis to elderly patients is lacking at present (16); 4) for the previous reasons we rather decided to express both the leucine and the glucose fluxes normalized for the ideal body weight of each individual subject as described in Methods.

It is important to note that neither young nor older

subjects were performing endurance training, since it is known that endurance training may affect leucine turnover (27). It would also have been very interesting to study an over-70 year old group to assess the role of further aging on the regulation of leucine metabolism by insulin and amino acids.

In conclusion, in middle-aged individuals we demonstrated a near-normal insulin- and amino acid-mediated protein metabolism, while an impairment of insulin-mediated glucose metabolism was already present. The physiological relevance of this early dissociation of insulin action on glucose and protein metabolism is noteworthy, since it may constitute an early defect modulating the changes of body composition and glucose tolerance of the elderly.

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