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Plasma acylcarnitines during insulin stimulation in humans are reflective of age-related metabolic dysfunction

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Abstract

The purpose of this study was to determine if plasma acylcarnitine (AC) profiling is altered under hyperinsulinemic conditions as part of the aging process. Fifteen young, lean (19–29 years) and fifteen middle- to older-aged (57–82 years) individuals underwent a 2-hr euglycemic-hyperinsulinemic clamp. Plasma samples were obtained at baseline, 20 min, 50 min, and 120 min for analysis of AC species and amino acids. Skeletal muscle biopsies were performed after 60 min of insulin-stimulation for analysis of acetyl-CoA carboxylase (ACC) phosphorylation. Insulin infusion decreased the majority of plasma short-, medium-, and long-chain (SC, MC, and LC, respectively) AC. However, during the initial 50 min, a number of MC and LC AC species (C10, C10:1, C12:1, C14, C16, C16:1, C18) remained elevated in aged individuals compared to their younger counterparts indicating a lag in responsiveness. Additionally, the insulin-induced decline in skeletal muscle ACC phosphorylation was blunted in the aged compared to young individuals (−24% vs. −56%, $P<0.05$). These data suggest that a desensitization to insulin during aging, possibly at the level of skeletal muscle ACC phosphorylation, results in a diminished ability to transition to glucose oxidation indicative of metabolic inflexibility.

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Keywords

acylcarnitine; acetyl-CoA carboxylase; amino acid; human; aging; metabolism

Introduction

Advancing age has been associated with a number of health-related issues including increased body fat [1], insulin resistance [2, 3], and type 2 diabetes (T2D) [4]. An index of metabolic health is metabolic flexibility which is the capacity of an organism to efficiently adjust substrate utilization to match fuel availability [5]. Research indicates that individuals who lack metabolic flexibility are more susceptible to health complications [5, 6]. Our previous research [2, 7] along with others [3] provide evidence that during hyperinsulinemia, aged individuals have impaired whole-body and skeletal muscle glucose metabolism. Despite this knowledge, the regulation and reliance on lipid metabolism during hyperinsulinemia, especially in the elderly, remains unclear and warrants further investigation.

Plasma acylcarnitines (AC) represent intermediates of fatty acid oxidation which cross the mitochondrial and cell membranes, enter circulation, and subsequently provide an index of mitochondrial beta-oxidation under a number of physiological conditions [6, 8, 9]. Increased fasting plasma AC species, especially long-chain AC (LC AC), have been observed in conditions of obesity [6, 10] and type 2 diabetes [6] suggesting dysregulation of fatty acid oxidation. Additionally, plasma AC and branched-chain amino acid signatures have been utilized as biomarkers for insulin resistance [9, 10] and help identify metabolic imbalances in fatty acid oxidation and amino acid catabolism. During insulin-stimulated conditions, plasma [8, 11] and skeletal muscle [12] AC concentrations have been reported to decline in healthy, insulin sensitive individuals, representing an efficient switch in substrate preference from lipid to carbohydrate oxidation. Conversely, in obese and type 2 diabetic individuals where metabolic flexibility is impaired, there is a blunted decline in AC species during hyperinsulinemic-euglycemic conditions [6], indicative of continued reliance on lipid oxidation.

While obesity and type 2 diabetes [6, 9, 10] have been examined in relation to AC profiling, it remains unclear if plasma AC profiling is altered with the aging process. Additionally, it remains unknown whether the time course of AC concentrations in response to hyperinsulinemia is reflective of metabolic inflexibility with the aging process. The purpose of the present study was to compare the AC profile in response to hyperinsulimia in younger versus middle- to older-aged individuals.

Material and Methods

Participants

Fifteen young (19–29 years) and fifteen middle- to older-aged (57–82 years) individuals were recruited from a larger study examining aging and insulin sensitivity [2]. Characteristics of the subjects are provided in Table 1. Briefly, all participants were non-

smokers and participated in less than 1h/week of organized physical activity. Body mass index (BMI) was between the 25th and 75th percentile of the United States population for the respective decade of age, in an attempt to study a representative population reflective of the “normal’ aging process [13]. Written informed consent was obtained and the protocol was in accordance of the Declaration of Helsinki and approved by the East Carolina University Policy and Review Committee on Human Research.

Preliminary Assessment

Aerobic capacity (VO2peak) was measured by expired gas analysis (TrueMax 2400; ParvoMedics, Sandy, UT) with an incremental maximal treadmill test [2]. Percent body fat was measured by dual X-ray absorptiometry.

Euglycemic-hyperinsulinemic clamp

Subjects reported to the laboratory at 0700 after a 12-h overnight fast. A 2-h euglycemic-hyperinsulinemic clamp was used to determine insulin sensitivity, as previously described [2, 14]. Blood plasma was obtained prior to insulin infusion and at 20 min, 50 min, and 120 min, and stored at −80°C for the subsequent analysis of non-esterified free-fatty acid (NEFA), acylcarnitines (AC), and amino acids. A biopsy specimen was obtained from the vastus lateralis with the percutaneous muscle biopsy technique at baseline and at 60 min of the clamp. Tissue samples were immediately frozen in liquid nitrogen for subsequent analyses of acetyl-CoA carboxylase (ACC) status.

Western blotting procedures

Skeletal muscle was homogenized and protein content was determined as previously described [2, 15] on all available muscle tissue (young, n=11; aged n=11). Cellular protein was separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes and probed overnight for phosphorylation of ACC on site Ser79 (Cell Signaling Technology, Danvers, MA). Samples were normalized to a control sample on each gel, and phosphorylation levels were additionally normalized to ACC total protein (Cell Signaling Technology, Danvers, MA) after membranes were stripped, as previously reported [2].

Plasma acylcarnitines, amino acids and NEFA

Acylcarnitines and amino acids were analyzed by flow injection tandem mass spectrometry using sample preparation methods described previously [16, 17]. The data were acquired using a Waters AcquityTM UPLC system equipped with a TQ (triple quadrupole) detector and a data system controlled by MassLynx 4.1 operating system (Waters, Milford, MA). (MS/MS) [9, 18]. NEFAs were assayed in duplicate on 5 μl serum diluted 1:1 with 0.9% saline using NEFA-HR(2) Kit Reagents (Wako Diagnostics, Mountain View, CA) in 96-well plate format.

Statistics

Analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL) and the R statistical language version 3.2. Pearson correlation coefficients and stepwise regression analysis were used to determine associations. Differences between young and

aged individuals at baseline were evaluated by unpaired t-test. To evaluate differences between young and aged individuals in response to insulin, a mixed-effects model was fit to each outcome. The model compared young and old group means using baseline values as a covariate. Data are presented as means ± SEM unless otherwise noted. Statistical significance was defined as $P < 0.05$.

Results

The middle- to older age individuals exhibited an elevated BMI, body fat percentage and fasting plasma glucose and insulin concentrations compared to their younger counterparts (Table 1). Insulin action (M-value) and VO2peak were lower in the aged subjects. The proportion of females was similar in both young and aged groups (27%). As reported previously [2], analysis of 3-day diet records revealed no significant dietary differences between the two age groups.

Fasting plasma acylcarnitines and amino acids

Fasting plasma NEFA (Table 1) and acetylcarnitine (C2) (Supplementary Material) levels did not differ between the two age groups. Aged individuals had higher fasting plasma C3 (Figure 1B), C4/Ci4 (Figure 1C), C10 (Figure 1D), C10:2 (Figure 1F), C14 (Figure 1H), C16 (Figure 1I), and lower C20:4 (Supplementary Material) levels relative to young individuals. When the data from both groups were combined, insulin action (M values) was inversely correlated with free carnitine ($r = -0.37$, $P<0.05$), C10 ($r = -0.39$, $P<0.05$), C14 ($r = -0.37$, $P<0.05$), C16:1 ($r = -0.37$, $P<0.05$) C16 ($r = -0.49$, $P<0.01$) and positively correlated with C5:1 ($r = 0.47$, $P<0.01$), C5 ($r = 0.51$, $P<0.01$), C20:4 ($r = 0.44$, $P<0.05$), C20 ($r = 0.38$, $P<0.05$). BMI was positively correlated with C3 ($r = 0.44$, $P<0.05$) and total percent body fat did not correlate with any fasting plasma AC levels.

In the fasted state, plasma amino acids glycine (Gly) and asparagine/aspartic acid (Asx) were higher in young individuals compared to aged individuals (13.6% and 90.2%, respectively, Figures 2A and 2B) while plasma citrulline (Cit) and arginine (Arg) were higher in aged individuals compared to their younger counterparts (38.8% and 16.3%, respectively, Figure 2C and Figure 2D). Plasma BCAA (leucine/isoleucine and valine) in the fasted state did not differ between the two age groups (Figures 2E and Supplementary Material). In the whole group, fasting plasma Cit was the only amino acid that was significantly related to insulin sensitivity ($r = -0.538$, $P<0.005$). Cit was positively correlated with total body fat ($r = 0.41$, $P<0.05$) and BMI ($r = 0.47$, $P<0.01$).

Insulin effect on plasma acylcarnitines and amino acids common between age groups

In the whole group, NEFA concentrations significantly decreased (20 min: −48%, 50 min: −82%, 120 min: −89%, $P<0.001$, Figure 1L) in response to insulin. Similarly, total short-chain (sum of C3, C4/Ci4, C5, C5:1; 20 min: 0%, 50 min: −6%, 120 min: −17%, $P<0.001$), medium-chain (sum of C6, C8, C8:1, C10, C10:1, C10:2, C10:3, C12, C12:1; 20 min: −18%, 50 min: −33%, 120 min: −47%, $P<0.001$) and long-chain acylcarnitines (sum of C14, C14:1, C14:2, C16, C16:1, C16:2, C18, C18:1, C18:2, C20, C20:4; 20 min: −6%, 50 min: −21%, 120 min: −40%, $P<0.001$) declined with insulin stimulation.

All individual saturated and unsaturated AC species between carbon lengths 2 and 20 decreased with insulin, regardless of age (Figure 1B–K), with the exception of C5:1 and C20 (Supplementary Material), which did not demonstrate a main effect for insulin. Insulin infusion had no effect on free carnitine (C0) (Figure 1A) or very-long acylcarnitine, C22 concentrations (Supplementary Material), in either group.

In the whole group, with the exception of plasma Asx and histidine (His) (Figures 2B and 2I) which did not change, all amino acids significantly declined during insulin infusion ($P<0.05$, Figure 2A and Figure 2C–H). Of the 13 amino acids that declined, leucine/isoleucine (Leu/Ile) demonstrated the greatest reduction (20 min: −9%, 50 min: −30%, 120 min: −56%, $P<0.001$, Figure 2E). Stepwise linear regression analysis determined that insulin sensitivity was the lone independent predictor of the decline in leucine/isoleucine (adjusted $R2 = 0.16$, $P<0.05$).

Age effect on insulin-induced decline of plasma acylcarnitines and amino acids

Although plasma SC, MC, and LC AC decreased in both groups in response to insulin, the pattern and magnitude of change for a number of individual AC species differed between the young and aged individuals, after correcting for baseline differences. After 20 minutes of insulin infusion, young individuals had significantly lower C10:1 (−30%, $P<0.05$, Figure 1E), C10 (−50%, P<001, Figure 1D), C12:1 (−46%, $P<0.001$, Figure 1G), C14 (−32%, $P<0.005$, Figure 1H), C18 (−30%, $P<0.05$, Figure 1K) and NEFA (−27%, $P<0.001$, Figure 1L) levels compared to aged individuals. After 50 minutes of insulin infusion, several LC AC were lower in the young compared to aged individuals including, C14 (−28%, $P<0.05$, Figure 1H), C16 (−14%, $P<0.05$, Figure 1I), C16:1 (−25%, $P<0.05$, Figure 1J), and C18 (−37%, $P<0.01$, Figure 1K), with C14 remaining lower (−38%, $P<0.05$, Figure 1H) 2 hours after insulin infusion. Age-related differences in the majority of MC and LC AC species remained even after controlling for differences in body fat and aerobic capacity. The AC, C3, commonly derived from BCAA catabolic pathways, was significantly higher in the aged group at all time points (Figure 1B). Aged individuals had a significantly higher (+27%, $P<0.05$, Figure 1A) plasma free carnitine after 120 minutes of insulin compared to their younger counterparts.

Plasma Cit and Arg were significantly higher in aged individuals (Figure 2C–D), whereas plasma Asx was higher in young individuals (Figure 2B), at all time points during insulin infusion, even after controlling for baseline differences. Plasma His was higher in aged individuals after 20 min of insulin-stimulation (Figure 2I). After 50 min of insulin infusion, plasma glutamine/glutamic acid (Glx, Figure 2G) and ornithine (Orn, Figure 2H) were higher in aged individuals, with ornithine remaining elevated at 120 min. Plasma phenylalanine (Phe) was elevated in the aged group at the end of the insulin infusion (Figure 2F). Young individuals had a blunted decline in plasma Gly after 20 min of insulin infusion compared to their aged counterparts (Figure 2A), but no differences were noted between the groups at later time points.

Skeletal muscle ACC phosphorylation during insulin infusion

To examine a possible mechanism contributing to the accumulation of LC AC during insulin stimulation in aged individuals, we measured changes in skeletal muscle ACC phosphorylation, an indirect marker of mitochondrial carnitine palmitoyltransferase I (CPT1) activity. In response to 60 min of hyperinsulinemia, the decline in ACC phosphorylation was greater in young individuals compared to aged individuals (−56% vs. −24%, $P<0.05$; Figure 3A). When the whole-group was included, there was a significant negative correlation ($r = -0.44$, $P<0.05$, Figure 3B) between whole-body insulin sensitivity and changes in skeletal muscle ACC phosphorylation. Despite no baseline differences in the plasma free carnitine/(C16+C18) ratio, a marker of CPT-1 function, young individuals had a greater increase (greater CPT-1 inhibition) compared to aged individuals (35% vs. 12%, $P<0.05$) after 50 min of insulin infusion (Figure 3C). Bivariate correlation analysis revealed that changes in skeletal muscle ACC phosphorylation were positively associated with changes in total LC AC ($r=0.54$, $P<0.01$, Figure 4A), but not total MC or SC AC (Figure 4B–C).

Discussion

Plasma AC levels have been previously shown to decline in healthy, lean individuals during physiological conditions that favor the switch from lipid to glucose utilization, including hyperinsulinemia [6, 11, 12]. In contrast, a blunted decline in plasma AC has been observed with obesity and/or T2D during insulin-stimulated conditions [6]. In the current study, the onset of insulin infusion elicited an overall decrease in plasma AC in both young and aged individuals (Figure 1). Plasma AC levels have been shown to reliably reflect fatty acid oxidation rates under a number of physiological conditions [6, 8, 9], therefore, it is highly likely the decline in plasma AC in the current study was indicative of the decreased lipid oxidation commonly associated with hyperinsulinemia [6, 17]. A critical finding in the present study was that a number of AC species remained elevated during hyperinsulinemia in the aged individuals compared to their younger counterparts, providing novel insight into a possible inefficiency in substrate switching, or metabolic inflexibility with age. Collectively, these data provide evidence that conditions of whole-body insulin resistance including obesity, T2D, and aging are linked to the inability to reduce lipid oxidation during conditions that favor glucose utilization.

The current study expanded on previous research examining metabolic flexibility by measuring plasma NEFA and AC concentrations at several time points during hyperinsulinemia in an attempt to discern if the time course, in addition to absolute concentrations, differed between the two age groups. Indicative of a delayed ability to adjust substrate availability, we observed a diminished decline in NEFA levels (Figure 1L) at the onset of the insulin infusion (20 min) in the aged group, implying that insulin was initially ineffective in inhibiting lipolysis. Elevations in lipolysis could suggest the mitochondria from older individuals had greater fatty acid availability during the onset of insulin-stimulation which could contribute, at least in part, to the continued entry of LC fatty acids into the mitochondria for beta-oxidation. Age-related elevations in plasma MC and LC AC were also evident within the first 50 minutes of the hyperinsulinemic-euglycemic clamp

(Figure 1), implying continued reliance on lipid oxidation and/or impaired complete lipid oxidation during this time period.

To our knowledge this is the first study to report elevated plasma MC and LC AC under fasting and insulin-stimulated conditions with the aging process. The present study also expands on previous studies by determining the elevation in LC AC species during insulin infusion in aged individuals was independent of initial baseline (fasting) differences (Figure 1). Investigating the effects of age are often complicated by the fact that aging is associated with changes known to contribute to metabolic dysfunction, such as increased body fat and reduced aerobic capacity (Table 1). An important finding in the current study was that fasting AC levels were not related to total percent body fat, and after controlling for body fat and aerobic capacity, age-related differences in insulin-stimulated AC species were still present (Figure 1).

A novel finding in the current study was that AC concentrations in older individuals normalized to their younger counterparts after 2 hours of hyperinsulinemia (Figure 1). Mihalik et al. [6] previously reported that plasma LC AC concentrations (C16, C18:1, C18) in obese/type 2 diabetics were elevated after 4 hours of hyperinsulinemic conditions compared to lean controls. While a direct comparison between our study and Mihalik et al. [6] is somewhat compromised due to differences in insulin dose (100 vs 40 mU/m2 per min), and only a single insulin-stimulated AC measurement in the latter, the findings from the two studies provide evidence that the underlying etiology and/or severity of the metabolic dysfunction observed with aging differs from insulin resistant conditions such as obesity or T2D.

The increased levels of LC AC species in the aged individuals during insulin stimulation implies that the early steps of beta-oxidation were elevated and fatty acid entry into the mitochondria was not limited during hyperinsulinemia. In an attempt to examine if the increased reliance on lipid oxidation in the aged group was due to a potential impairment at the level of fatty acid entry into the mitochondria, we measured the phosphorylation status of ACC in skeletal muscle.

Insulin is known to dephosphorylate ACC, thereby increasing ACC activity and increasing the production of malonyl-CoA [19], resulting in the inhibition of CPT1 and thus fatty acid oxidation. Similar to findings in insulin-resistant obese and type 2 diabetics [19], our results demonstrate that aged individuals have an impaired ability to dephosphorylate ACC (indicating increased ACC activity) in skeletal muscle during the first hour of the hyperinsulinemic-euglycemic clamp (Figure 3A). This impaired ability to dephosphorylate ACC would allow CPT1 activity to remain elevated. Of interest, the ability of insulin to dephosphorylate skeletal muscle ACC was related to the decline in LC AC, suggesting the more pronounced decline in ACC phosphorylation in our young individuals may have played a role in inhibiting LC fatty acid entry into the mitochondria for oxidation. Our group recently reported that insulin-stimulated PDH activity was reduced in the skeletal muscle of middle to older aged individuals [7], suggesting the ability to increase skeletal muscle glucose oxidation during hyperinsulinemia may also be compromised with age. Collectively, these findings suggest that aging skeletal muscle loses the ability for insulin to stimulate key

metabolic proteins responsible for inhibiting lipid and promoting glucose entry into the mitochondria for oxidation. While we acknowledge a number of tissue are responsible for the appearance of plasma metabolites, it is possible that age-related metabolic dysfunction in skeletal muscle may contribute, at least in part, to the elevated plasma AC observed in the current study.

Clinical studies investigating obesity have reported a positive relationship between plasma branched chain amino acids (BCAA) and insulin resistance [10, 20]. In contrast to these studies, aged individuals had similar fasting levels of plasma BCAA (i.e, isoleucine/leucine) as their younger counterparts and despite age-related differences in AC C3, a by-product of BCAA catabolism (Figure 1B) the plasma BCAA response to insulin was similar between the two age groups (Figure 2E). Together, these data indicate that despite the apparent correlation between elevated BCAA metabolism and insulin resistance in obese populations, BCAA metabolism appears to be largely unaffected in insulin-resistant aged individuals.

In summary, AC profiling reveals inefficiencies indicative of metabolic inflexibility in aged, sedentary individuals compared to their younger counterparts. The blunted decline in LC AC in response to insulin was related to the inability to dephosphorylate ACC in skeletal muscle. Although AC concentrations differed initially in response to insulin infusion in aged individuals, many of the differences eventually normalized to those seen in young individuals suggesting that although metabolic inflexibility is evident with older adults, it may not be as progressive as with other insulin-resistant conditions such as T2D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Plasma acylcarnitine profiling reveals metabolic inflexibility in aged individuals.
- Time course acylcarnitine profiling is critical to identify metabolic dysfunction.
- Acetyl-CoA carboxylase phosphorylation status is related to metabolic dysfunction.

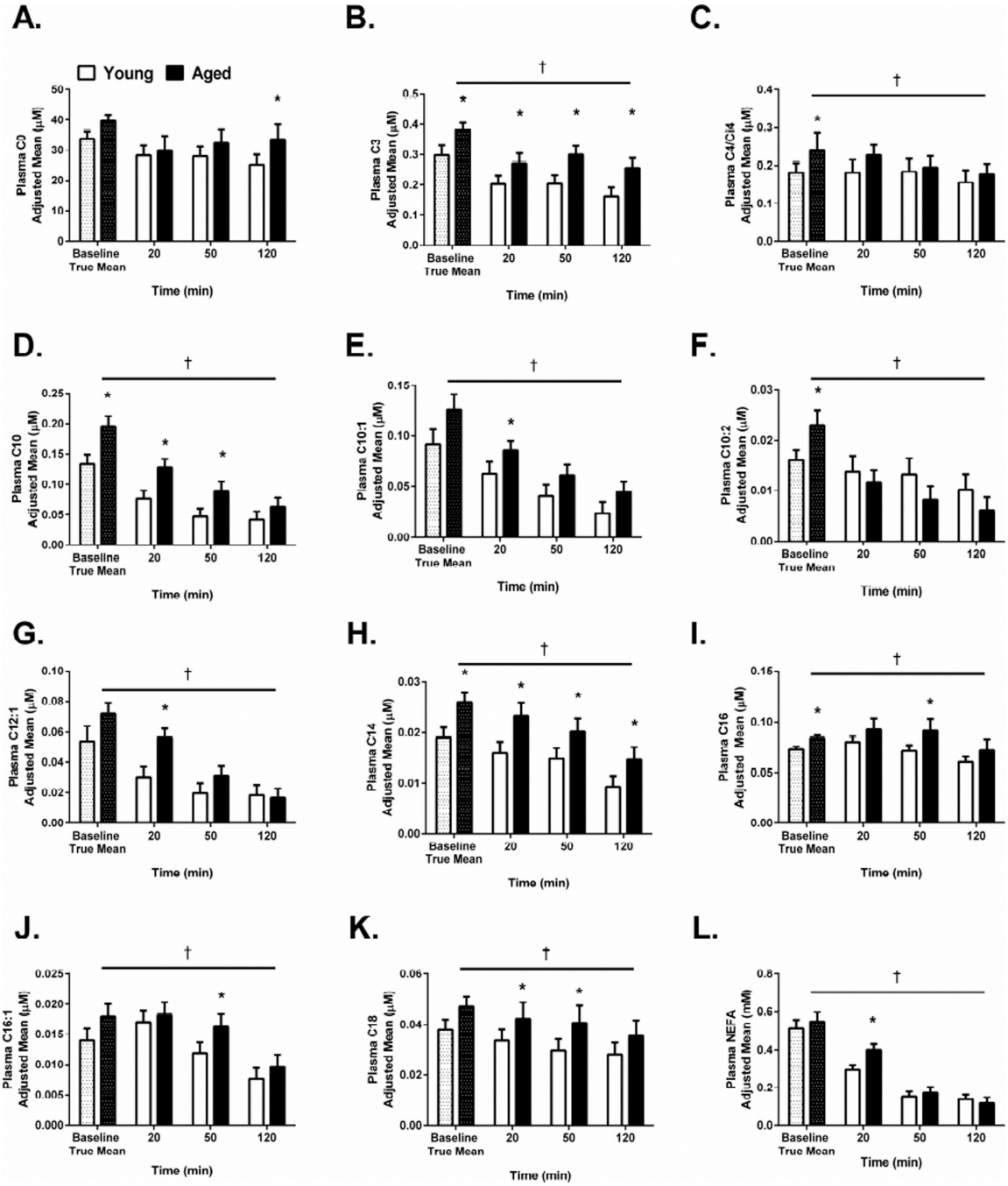

Figure 1. Plasma free carnitine, acylcarnitine and NEFA concentrations during fasting and the hyperinsulinemic-euglycemic clamp in young (n=15) and aged (n=15) individuals

Data are presented as true means or adjusted means ± SEM. Differences between age groups at baseline were determined by unpaired t-test. Differences between age groups during insulin infusion were determined using a repeated-measures ANCOVA, with baseline differences as a covariate. Hatched bars indicate true baseline mean and solid bars represent adjusted means determined by ANCOVA. * Significantly different from young individuals ($P<0.05$). †Significant main effect for time ($P<0.05$).

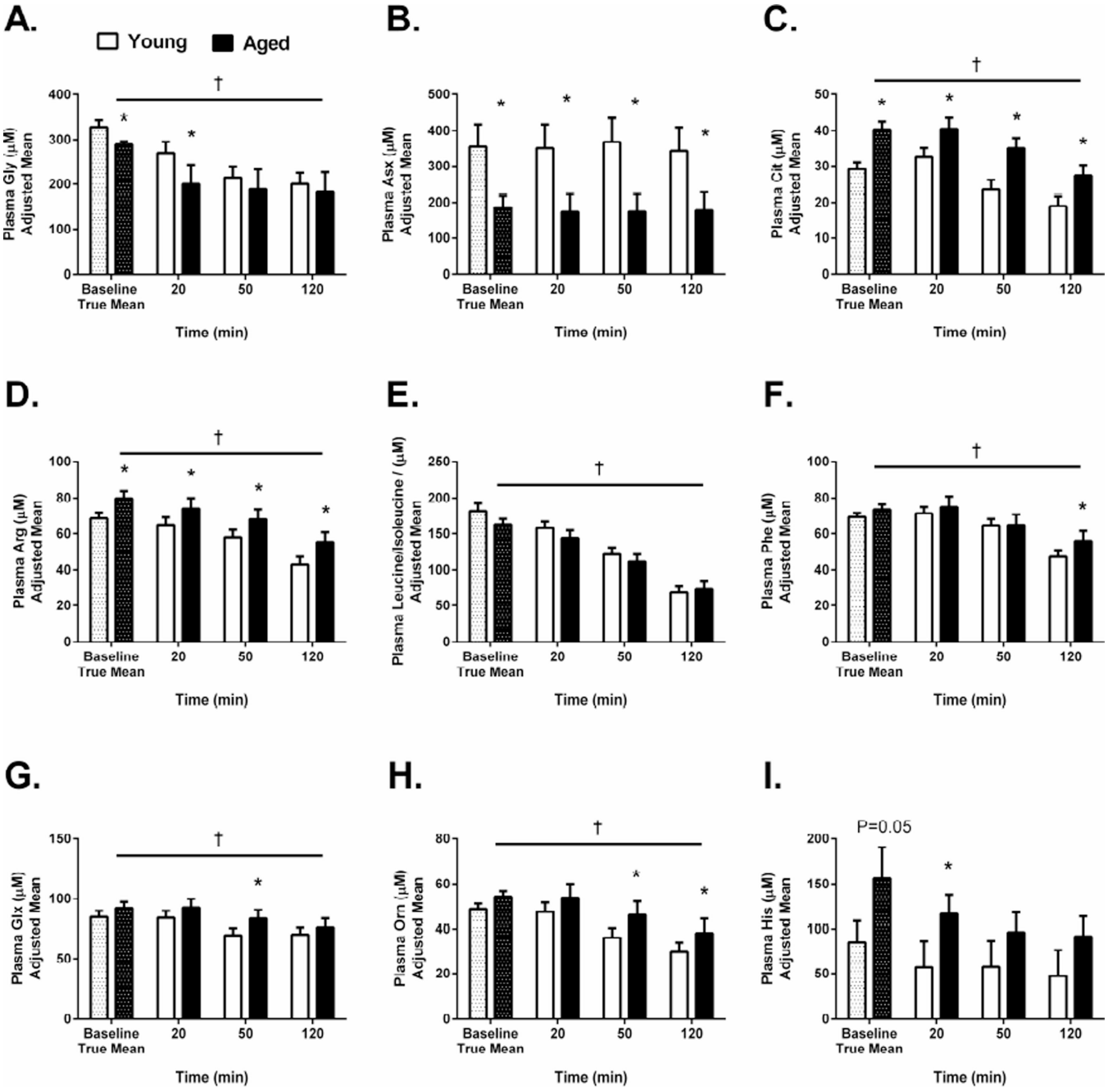

Figure 2. Plasma amino acids during fasting and the hyperinsulinemic-euglycemic clamp in young (n=15) and aged (n=15) individuals

Data are presented as true means or adjusted means ± SEM. Differences between age groups at baseline were determined by unpaired t-test. Differences between age groups during insulin infusion were determined using a repeated-measures ANCOVA, with baseline differences as a covariate. Hatched bars indicate true baseline mean and solid bars represent adjusted means determined by ANCOVA. * Significantly different from young individuals (P<0.05). †Significant main effect for time (P<0.05).

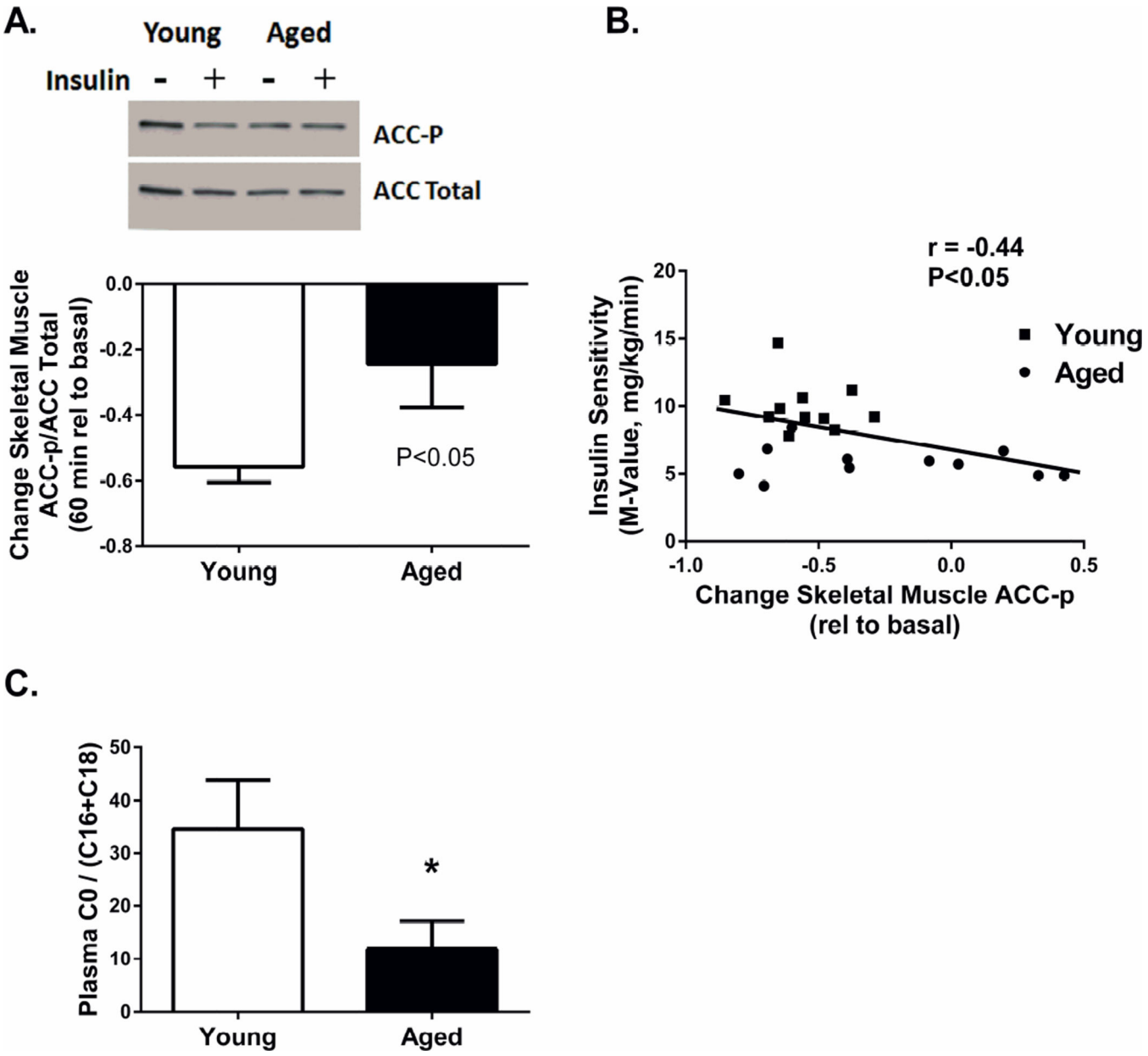

Figure 3. Skeletal muscle and plasma markers indicate impaired inhibition of ACC and CPT-1 in aged individuals in response to insulin infusion

Skeletal muscle ACC phosphorylation after 60 min of insulin infusion in young (n=11) and aged individuals (n=11) (A). Data was then presented as ACC phosphorylation at 60 min relative to basal levels. Data are presented mean ± SEM. Correlational analysis between whole-body insulin sensitivity (M-value) and the change in skeletal muscle ACC phosphorylation after 60 minutes of insulin infusion (relative to basal levels) in the whole study group (n=22) (B). Plasma ratio of free carnitine relative to C16+ C18 at 50 min of insulin infusion in young (n=15) and aged individuals (n=15) (C).

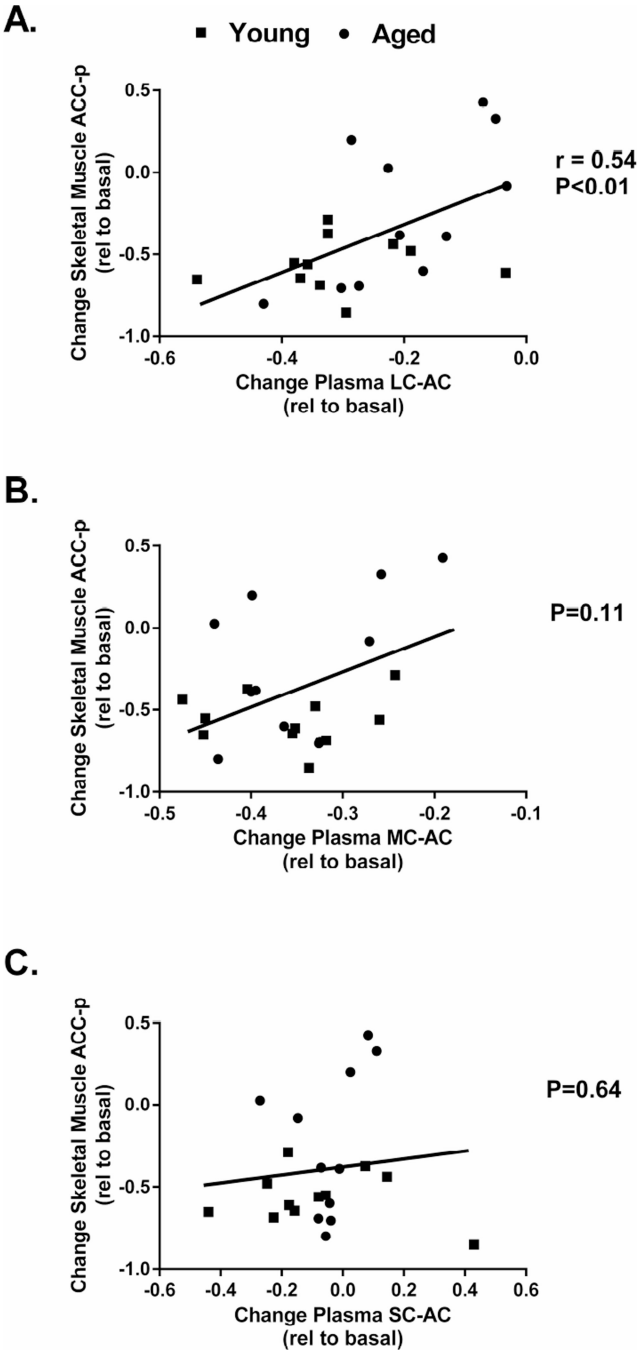

Figure 4. Correlational analysis between changes in skeletal muscle ACC phosphorylation and plasma acylcarnitines in the whole-study group (n=22)

Correlational analysis between changes in skeletal muscle ACC phosphorylation and changes in the sum of plasma LC acylcarnitines (A), MC acylcarnitines (B) and SC acylcarnitines (C). Skeletal muscle ACC phosphorylation was measured after 60 min of insulin infusion and plasma acylcarnitines were measured after 50 min of insulin infusion. Both muscle and plasma values were presented relative to basal levels.

Table 1

Participant Demographics

	Young Individuals	**Aged Individuals**
Age	22.1 ± 2.7	69.8 ± 7.9***
Gender (m/f)	11/4	11/4
Insulin Action (M-value)	9.6 ± 2.0	5.6 ± 1.3***
BMI (kg/m2)	22.4 ± 2.2	28.9 ± 3.7***
Body Fat (%)	19.0 ± 8.7	34.8 ± 6.2***
VO2peak (ml/kg/min)	40.8 ± 8.4	21.3 ± 4.6***
Fasting plasma glucose (mg/dL)	81.7 ± 6.2	90.5 ± 8.2**
Fasting plasma insulin (mIU/mL)	3.7 ± 3.0	6.2 ± 3.7*
Fasting plasma NEFA (μmol/l)	512.1 ± 178.1	549.4 ± 181.8
HOMA-IR	1.0 ± 0.7	1.6 ± 0.7

*** P<0.001,

** P<0.005

* P<0.05