

## High Insulin Combined With Essential Amino Acids Stimulates Skeletal Muscle Mitochondrial Protein Synthesis While Decreasing Insulin Sensitivity in Healthy Humans

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**Context:** Insulin and essential amino acids (EAAs) regulate skeletal muscle protein synthesis, yet their independent effects on mitochondrial protein synthesis (MiPS) and oxidative function remain to be clearly defined.

**Objective:** The purpose of this study was to determine the effects of high or low insulin with or without EAAs on MiPS.

**Design:** Thirty participants were randomized to 3 groups of 10 each with each participant studied twice. Study groups comprised (1) low and high insulin, (2) low insulin with and without EAAs, and (3) high insulin with and without EAAs.

**Setting:** The study was conducted in an in-patient clinical research unit.

**Participants:** Eligible participants were 18 to 45 years old, had a body mass index of  $<25 \text{ kg/m}^2$ , and were free of diseases and medications that might impair mitochondrial function.

**Intervention:** Low ( $\sim 6 \mu\text{U/mL}$ ) and high ( $\sim 40 \mu\text{U/mL}$ ) insulin levels were maintained by iv insulin infusion during a somatostatin clamp while maintaining euglycemia ( $4.7\text{--}5.2 \text{ mM}$ ) and replacing GH and glucagon. The EAA infusion was 5.4% NephroAmine.  $\text{L-}[\text{ring-}^{13}\text{C}_6]\text{Phenylalanine}$  was infused, and muscle needle biopsies were performed.

**Main Outcomes:** Muscle MiPS, oxidative enzymes, and plasma amino acid metabolites were measured.

**Results:** MiPS and oxidative enzyme activities did not differ between low and high insulin (MiPS:  $0.07 \pm 0.009$  vs  $0.07 \pm 0.006\%/h$ ,  $P = .86$ ) or between EAAs and saline during low insulin (MiPS:  $0.05 \pm 0.01$  vs  $0.07 \pm 0.01$ ,  $P = .5$ ). During high insulin, EAAs in comparison with saline increased MiPS ( $0.1 \pm 0.01$  vs  $0.06 \pm 0.01$ ,  $P < .05$ ) and cytochrome c oxidase activity ( $P < .05$ ) but not citrate synthase ( $P = .27$ ). EAA infusion decreased ( $P < .05$ ) the glucose infusion rates needed to maintain euglycemia during low ( $\sim 40\%$ ) and high insulin ( $\sim 24\%$ ).

**Conclusion:** EAAs increased MiPS and oxidative enzyme activity only with high insulin concentrations. (*J Clin Endocrinol Metab* 99: E2574–E2583, 2014)

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Abbreviations: COXIV, cytochrome c oxidase IV; EAA, essential amino acid; FFM, fat-free mass; FSR, fractional synthesis rate; GC, gas chromatography; GIR, glucose infusion rate; MITO, mitochondrial; MMP, mixed muscle protein; MPE, molar percent excess; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MYO, myofibrillar; Phe  $R_a$ , phenylalanine rate of appearance; RER, respiratory exchange ratio; SARC, sarcoplasmic;  $\text{Vco}_2$ , carbon dioxide consumption;  $\text{Vo}_2$ , oxygen consumption.

Skeletal muscle protein content depends on the net contribution of protein synthesis and degradation. Skeletal muscle protein synthesis is commonly measured using mixed muscle homogenates, yet isolation techniques reveal that rates vary between specific organelles and individual proteins (1). The synthesis of many muscle proteins declines with chronic conditions such as aging (2, 3), and understanding the factors regulating fractional synthesis rates (FSRs) of muscle proteins with specific functions will help target future therapies. Insulin and amino acids regulate mixed muscle protein (MMP) synthesis, but their impact on protein fractions, particularly the mitochondria, is less known. Mitochondrial protein content and function decline with aging (3) and type 2 diabetes (4), including impaired insulin and amino acid signaling (5). Yet, the roles of insulin and amino acids in maintaining the mitochondrial proteome are not fully elucidated.

The relationship between plasma insulin and amino acid concentrations makes it difficult to discern their individual effects. Essential amino acids (EAAs) are required for protein synthesis and are provided through the diet or after degradation of endogenous proteins. Raising plasma insulin to 15 to 30  $\mu$ U/mL suppresses protein degradation with no further suppression at higher concentrations (6, 7). Accordingly, EAA concentrations decrease during insulin infusion with no stimulation of mixed muscle (8, 9) or mitochondrial protein synthesis (10). Previous studies reported that raising insulin while maintaining amino acid concentrations increased the mRNA markers of mitochondrial biogenesis and protein synthesis (11). Yet the independent effect of amino acids on mitochondrial function has yielded mixed results. Infusion of branched-chain amino acids into humans increased skeletal muscle mitochondrial activity, which suggests a beneficial role for stimulating mitochondrial function (12). However, in miniature swine, insulin infusion stimulated muscle mitochondrial protein synthesis with no further increase during amino acid infusion (13). It remains to be determined whether mitochondrial protein synthesis is stimulated by EAAs independent of insulin in humans and whether the effect depends on the dose of EAAs or insulin.

Amino acids and metabolites are implicated in the development of insulin resistance. An important question is whether addition of EAAs to the infusion, as occurs after a mixed meal, may cause insulin resistance and whether higher insulin is needed to overcome this resistance. People with insulin resistance are more likely to have elevated branched-chain amino acid metabolites, and supplementing branched-chain amino acids to a high-fat diet increased insulin resistance in mice (14, 15). Branched-chain amino acids are oxidized in skeletal muscle mitochondria, and increased respiratory chain flux may impair insulin

signaling due to increased oxidative stress (15). The ability for amino acids to induce insulin resistance must be considered in combination with mitochondrial protein synthesis (16).

In the current study, we sought to determine mitochondrial protein synthesis in response to low- and high-dose insulin in combination with EAA infusion. We performed euglycemic clamps and maintained insulin concentrations with iv infusion of insulin and somatostatin. EAAs were infused during low (fasting) and high (postprandial) concentrations of insulin. The primary hypothesis was that EAA infusion would increase mitochondrial protein synthesis during either low- or high-insulin conditions.

## Materials and Methods

The Mayo Clinic Institutional Review Board approved the study, and participants provided written informed consent. Inclusionary criteria were age of 18 to 45 years and body mass index of  $<25$  kg/m<sup>2</sup> (Table 1). Exclusionary criteria included diabetes mellitus, fasting glucose of  $>110$  mg/dL, active cardiovascular disease, liver disease, kidney failure, active endocrinopathies, chronic neurological diseases, stroke, myopathies, or metabolic conditions affecting the outcome measures. Participants taking medicines such as  $\beta$ -blockers or preparations that impair mitochondrial function were excluded. A screening visit included physical examination, blood draw for complete blood count, comprehensive metabolic panel, dietitian consult, and dual x-ray absorptiometry for body composition (QDR-4500; Hologic Inc). Participants ( $n = 30$ ) were randomly assigned to 1 of 3 study groups ( $n = 10$  each) and underwent 2 study days to compare iv infusion of (1) low insulin and high insulin, (2) low insulin + saline and low insulin + EAAs, or (3) high insulin + saline and high insulin + EAAs. Study days were separated by at least 2 weeks.

Each participant followed a weight-maintaining diet (50% carbohydrate, 30% fat, and 20% protein) for 3 days and was admitted to the Clinical Research Unit at the Mayo Clinic on the evening of the third day. At 5:15 AM on the study day, catheters were inserted into a dorsal hand vein for arterialized blood samples and into an antecubital vein on the contralateral arm for infusion. Infusions started at 6:00 AM for all studies: somatostatin (7  $\mu$ g/kg fat-free mass [FFM]/h), human GH (5 ng/kg FFM/

**Table 1.** Participant Characteristics

	Low vs High Insulin	Low Insulin $\pm$ EAAs	High Insulin $\pm$ EAAs
No. (male/female)	10 (5/5)	10 (5/5)	10 (5/5)
Age, y	28 $\pm$ 3	28 $\pm$ 8	27 $\pm$ 2
Body mass index, kg/m <sup>2</sup>	24.0 $\pm$ 0.6	23.4 $\pm$ 0.7	23.4 $\pm$ 0.7
Fat mass, %	30 $\pm$ 4.8	30 $\pm$ 4.2	29 $\pm$ 3.1
FFM, kg	44.6 $\pm$ 4	46.9 $\pm$ 4.4	48.0 $\pm$ 3.7
Fasting insulin, $\mu$ U/mL	4.8 $\pm$ 1	4.9 $\pm$ 0.5	3.7 $\pm$ 0.4
Fasting glucose, mg/dL	93 $\pm$ 1	93 $\pm$ 1	91 $\pm$ 2

ANOVA revealed no differences between groups in anthropometrics. Data are means  $\pm$  SEM.

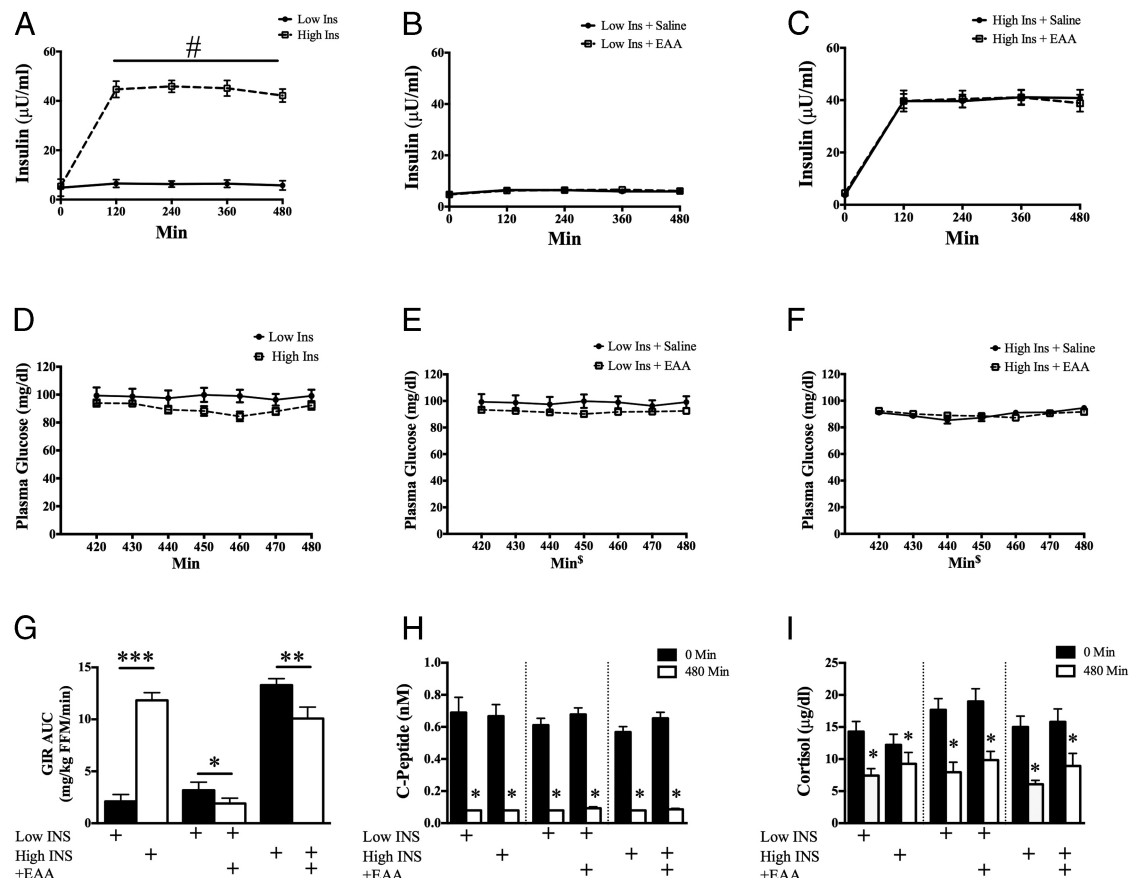
min), and a prime (1 mg/kg FFM) and an infusion (1 mg/kg FFM/h) of L-[ring- $^{13}\text{C}_6$ ]phenylalanine (Cambridge Isotope Laboratories). Study-specific infusions of insulin and EAAs started at 6:00 AM and continued for 480 minutes. Low insulin was maintained by iv infusion of insulin at 0.25 mU/kg FFM/min and high insulin at 1.5 mU/kg FFM/min. The EAA infusion was 5.4% NephroAmine at 3 mL/kg FFM/h. Plasma glucose was measured in duplicate every 10 minutes, and euglycemia (85–95 mg/dL) was maintained by variable infusion of 40% dextrose. Percutaneous muscle biopsy specimens of the vastus lateralis (~300 mg) were performed at 180 and 480 minutes under local anesthesia (2% lidocaine). Muscle samples were blotted, were dissected free of fat and connective tissue, and then were frozen. Biopsy specimens were collected from the opposite legs on a single visit. Indirect calorimetry was performed at 11:00 AM for 20 minutes using a ventilated hood (Parvo Medics). The final 10 minutes of steady-state oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) determined the respiratory exchange ratio ( $\text{RER} = \text{VCO}_2/\text{VO}_2$ ). Urine was collected throughout the clamp and analyzed for urea content at a core laboratory. Substrate oxidation and nonprotein RER were calculated as described previously (17).

MMP and muscle fractions were isolated from frozen samples using differential centrifugation (18, 19). Biopsy samples were homogenized with protease and phosphatase inhibitors (Halt; Thermo Fisher Scientific) and centrifuged to pellet myofibrillar (MYO) proteins. The supernatant was centrifuged to pellet mi-

tochondrial (MITO) proteins, and the final supernatant was deproteinized with cold ethanol (1:9, v/v) and then centrifuged to pellet sarcoplasmic (SARC) proteins. Aliquots from MMP, MYO, SARC, and MITO were acid hydrolyzed, and free amino acids were purified using cation exchange columns and then were dried.

Plasma phenylalanine enrichment was determined using gas chromatography (GC) and mass spectrometry (MS) as described previously (19). Samples were derivatized to a heptafluorobutyl isobutyl ester and identified with a Micromass Quattro Micro triple quadrupole GC-MS system (Waters) operating under negative ion chemical ionization using isobutane as the reactant gas. Selected ion monitoring of  $m/z$  399.2 and 403.2  $M + 2$  and  $M + 6$  fragments of phenylalanine and the L-[ring- $^{13}\text{C}_6$ ]phenylalanine, respectively, was performed.

Muscle protein enrichment was determined using HPLC and tandem mass spectrometry (MS/MS) as described previously (19). HPLC-MS/MS has superior precision comparable to those of isotope ratio mass spectrometry and GC-MS and accuracy comparable to that of isotope ratio mass spectrometry (19). Dried amino acids were prepared as isobutyl ester derivatives, and the mass was determined by an API 5000 triple quadrupole mass spectrometer with a TurbolonSpray source (Applied Biosystems). Enrichment was calculated as molar percent excess (MPE) representing tracer content ( $^{13}\text{C}$ ) over background. A calibration curve from 0 to 0.1 MPE was prepared using unlabeled L-phenylalanine (Sigma-Aldrich) and L-[ring- $^{13}\text{C}_6$ ]phenylalanine. Selected reaction monitor-



**Figure 1.** Plasma insulin (Ins) during clamp (A–C) and glucose concentration during the final 60 minutes of clamp (D–F). G, GIR between 420 and 480 minutes. Plasma C-peptide (H) and cortisol (I) were decreased from baseline to 480 minutes. #,  $P < .05$  for ANOVA, main effect of low vs high insulin study day. \*,  $P < .05$ ; \*\*,  $P < .01$ ; and \*\*\*,  $P < .001$  for paired  $t$  test of GIR within each group. \$,  $P < .05$  for ANOVA, main effect of time for plasma glucose. \*,  $P < .05$  for ANOVA, main effect of time for C-peptide and cortisol. Data are means  $\pm$  SEM from  $n = 10$  per group.

ing was performed for  $m/z$  222.4 > 121.6 and 226.4 > 125.6 for the  $M + 2$  and  $M + 6$  fragments of phenylalanine and L-[ring- $^{13}\text{C}_6$ ]phenylalanine, respectively.

Muscle FSRs were calculated as FSR (percentage per hour) =  $[(E_{480} - E_{180})/(E_p \cdot t)] \cdot 60 \cdot 100$ , with protein enrichment of biopsy samples ( $E_{480}$  and  $E_{180}$ ), minutes between biopsies ( $t$ ), and enrichment ( $E_p$ ) of plasma precursor (10). The phenylalanine rate of appearance (Phe  $R_a$ ) was calculated as  $\text{Phe } R_a = [(F_{\text{Tracer}}/E_p) - F_{\text{EAA}}]$ , with tracer infusion rate ( $F_{\text{Tracer}}$ ), plasma enrichment ( $E_p$ ), and phenylalanine appearance from EAA infusion ( $F_{\text{EAA}}$ ). One participant showed decreased plasma enrichment (10.4%–2.2%) during high insulin + saline for unknown reason and was removed from the protein turnover results. The muscle tissue required for fractionation was not available for all participants, and group sizes were 8 to 10 per fraction.

Citrate synthase activity was measured as described previously (20). The activity of mitochondrial complex IV was measured by immunocapturing cytochrome *c* oxidase IV (COXIV) and determining maximal enzyme activity to reduce cytochrome *c* normalized to enzyme content (Abcam).

Hormones were analyzed in the Immunochemical Core Laboratory at the Mayo Clinic using plasma from sodium-heparin tubes. Insulin was determined by a 2-site immunoenzymatic assay using a UniCel DxI 800 automated system (Beckman Instruments). C-peptide was determined using a 2-site immunometric assay (catalog number 03184897190; Roche Diagnostics) and detected using electrochemiluminescence detection (Roche Cobas e411; Roche Diagnostics). Human GH was measured using an immunoenzymatic assay (catalog number 33580; Beckman Coulter Inc). Glucagon was detected using a radioimmunoassay kit (GL-32K; Millipore Corp).

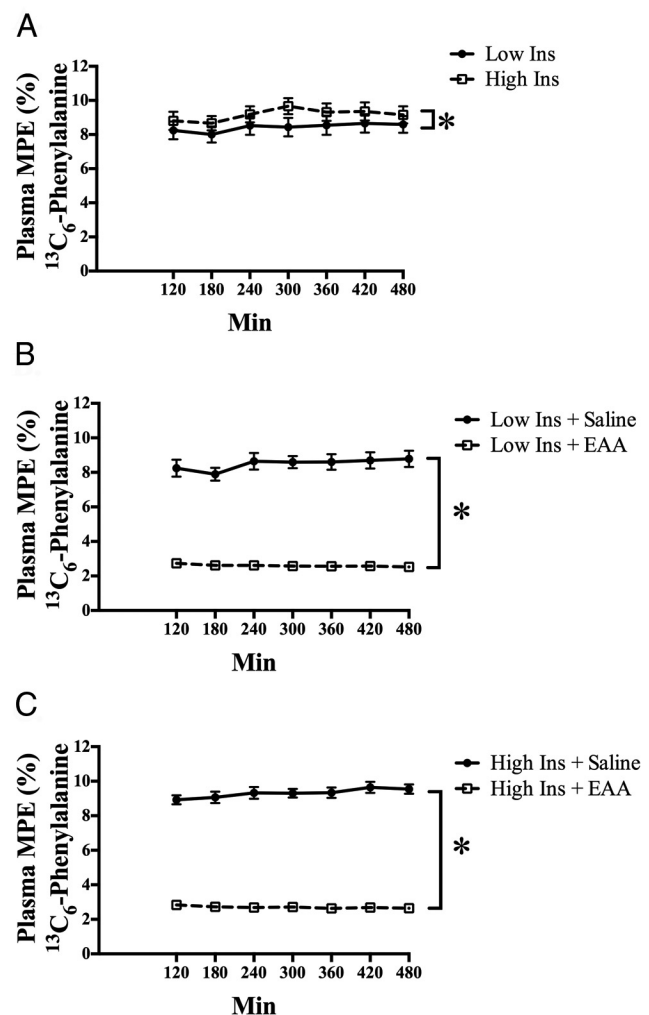
Plasma amino acids and metabolites were determined using MassTrak Amino Acid Solution (Waters) modified for MS (21). Samples were spiked with internal standards for amino acids and metabolites, deproteinized using cold methanol, and centrifuged. An aliquot of the supernatant was derivatized using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and separated with an Acquity ultraperformance liquid chromatograph, and mass detection was performed using a TSQ Ultra 182 Quantum mass spectrometer (Thermo Finnigan) in electrospray ionization positive mode.

Preliminary data indicated that a 20% effect size for the primary endpoint (mitochondrial protein synthesis) was approximately 0.0152%/h. The power for a two-sided test with  $\alpha$  at .05 and sample size of 10 per group is >99%. Each study group addressed a distinct experimental question, and the primary endpoint (protein synthesis) was tested individually for each group using paired *t* tests without multiple comparisons. The secondary endpoints including glucose infusion rate (GIR), Phe  $R_a$ , and indirect calorimetry were compared with paired *t* tests within a group. Within each group, plasma glucose, insulin, phenylalanine enrichment, amino acids, and metabolites were compared using a two-way ANOVA (study day  $\times$  time) with repeated measures and Sidak correction for multiple comparisons. Plasma values less than the level of detection were assigned the lowest value of the standard curve (0.08 mM for C-peptide and 0.5  $\mu\text{M}$  for 1- and 3-methylhistidine) and analyzed using a nonparametric Friedman test with Dunn adjustment for multiple comparisons. Significance was set as a value of  $P = .05$ . Data were analyzed using Prism 6 (GraphPad Software Inc) and are displayed as means  $\pm$  SEM.

## Results

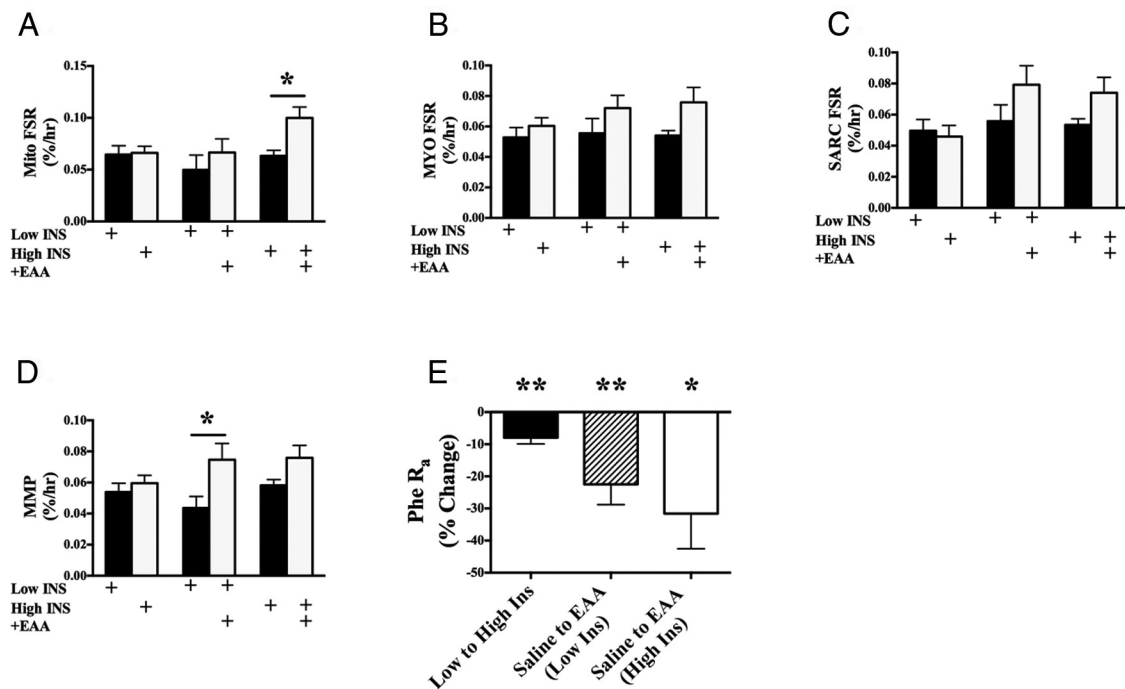
Low insulin maintained postabsorptive insulin concentrations, whereas high insulin maintained postprandial insulin concentrations (Figure 1, A–C). Euglycemia was maintained (Figure 1, D–F) between study visits. The exogenous GIR was greater ( $P < .01$ ) during high-dose insulin but lower ( $P < .05$ ) with EAA infusion during low or high insulin infusion (Figure 1G). C-peptide was suppressed by 480 minutes in all groups (Figure 1H), and cortisol was decreased at 480 minutes (Figure 1I). Glucagon ( $\sim 30$  pg/mL) and human GH ( $\sim 0.5$  ng/mL) were constant during the clamp (data not shown).

Plasma L-[ring- $^{13}\text{C}_6$ ]phenylalanine enrichment was maintained at steady state (slope of  $<0.01$ ) and was lower during high-dose insulin (Figure 2A) and infusion of non-enriched EAA solution (Figure 2, B and C). The slope of plasma enrichment was not different from 0 for all con-



**Figure 2.** Plasma isotopic enrichment of L-[ring- $^{13}\text{C}_6$ ]phenylalanine expressed as the MPE for low vs high insulin (Ins) (A), low insulin  $\pm$  EAAs (B), and high insulin  $\pm$  EAAs (C). \*,  $P < .05$  for ANOVA difference between study days. Data are means  $\pm$  SEM from  $n = 9$  to 10 per group.





**Figure 3.** Skeletal muscle FSR of MITO (A), MYO (B), SARC (C), and MMP (D). E, Phe  $R_a$  is displayed as percent change between paired study days. Data are means  $\pm$  SEM from  $n = 8$  to 10 per group. \*,  $P < .05$ ; and \*\*,  $P < .001$  for paired  $t$  test within each group. INS, insulin.

ditions except low insulin + EAAs (95% confidence interval  $-0.0007$  to  $-0.00006$ ), but the deviation was within tolerance of steady state.

MITO FSR was not changed by high insulin alone or EAAs with low insulin but increased with EAAs during high insulin (Figure 3A). MYO FSR was not changed during high insulin alone or with EAAs during low insulin but tended ( $P = .055$ ) to be higher with EAAs during high insulin (Figure 3B). SARC was not changed during any condition (Figure 3C). MMP was not changed by high insulin alone but increased with EAAs during low insulin and tended ( $P = .1$ ) to increase with EAAs during high insulin (Figure 3D). The Phe  $R_a$  from endogenous protein degradation (micrograms per kilogram of FFM per hour) (Figure 3E) decreased from low to high insulin ( $67.8 \pm 4.4$  vs  $61.9 \pm 3.2$ ,  $P < .05$ ). EAAs decreased Phe  $R_a$  during low insulin ( $64.6 \pm 3.3$  vs  $49.7 \pm 4.2$ ,  $P < .001$ ) and high insulin ( $63.4 \pm 4.8$  vs  $41.5 \pm 5.8$ ,  $P < .001$ ).

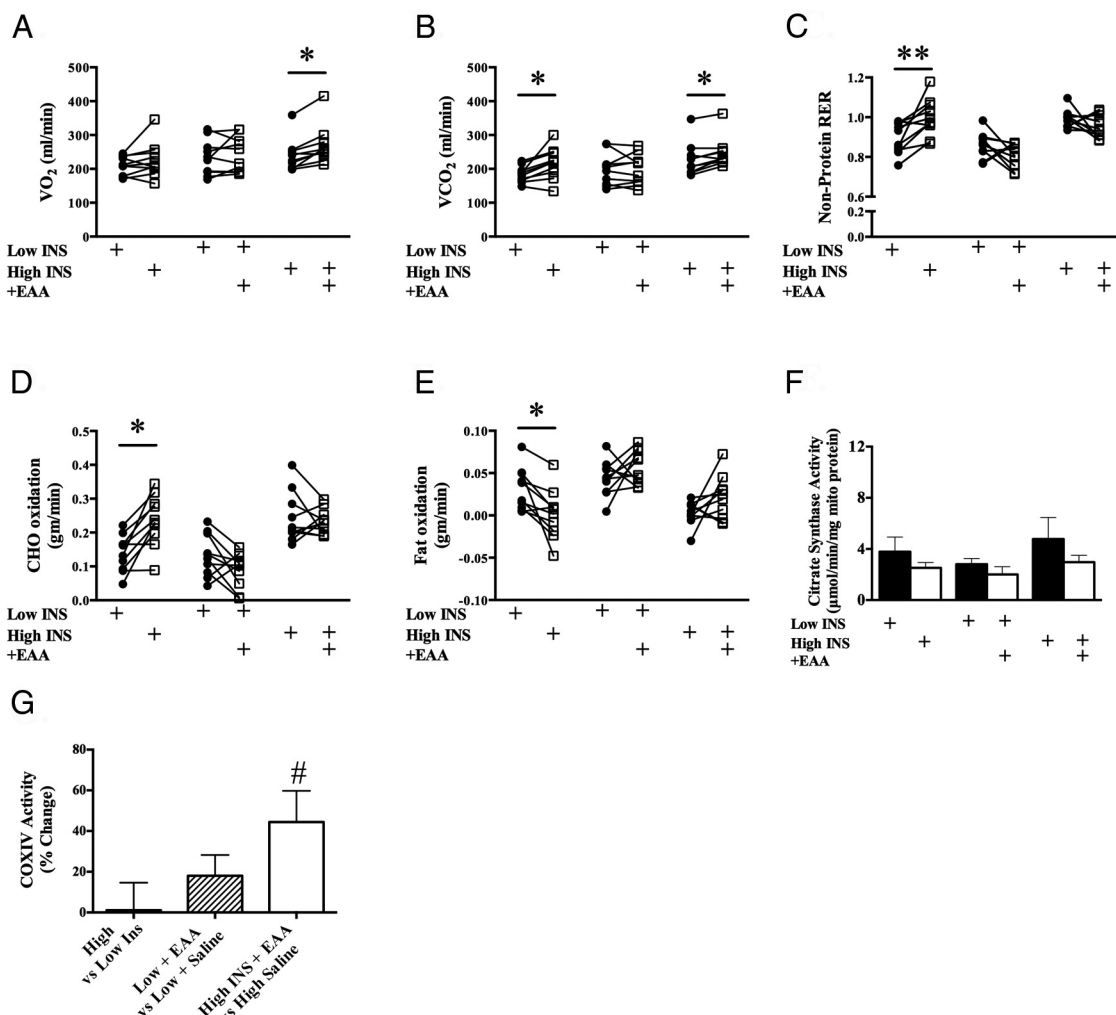
Increasing insulin from low to high increased  $V_{CO_2}$  without changing  $VO_2$  and contributed to higher nonprotein RER. Addition of EAAs to low insulin did not change  $V_{CO_2}$ ,  $VO_2$ , or nonprotein RER. Addition of EAAs to high insulin increased  $V_{CO_2}$  and  $VO_2$  while maintaining nonprotein RER (Figure 4, A–C). From low to high insulin, whole-body carbohydrate oxidation increased, whereas fat oxidation decreased. Whole-body substrate oxidation did not change with EAAs added to low or high insulin (Figure 4, D and E). The maximal activity of citrate synthase was not changed during any condition (Figure 4F).

COXIV activity was stimulated  $\sim 44\%$  when EAAs were added to high insulin (Figure 4G).

Branched-chain amino acids and EAAs decreased from low to high insulin and increased during EAA infusion during low and high insulin (Figure 5, A–C, and Supplemental Table 1). The increase in branched-chain amino acids was lower during high insulin vs low insulin ( $P < .001$ , unpaired  $t$  test). The lysine metabolite  $\alpha$ -amino adipic acid decreased during low and high insulin and then increased during EAA infusion regardless of insulin concentrations (Figure 5D). High insulin decreased  $\alpha$ - and  $\beta$ -aminoisobutyric acid, whereas EAA infusion increased the concentrations above baseline during low insulin and maintained the concentrations at baseline during high insulin (Figure 5, E and F). Plasma 1- and 3-methylhistidine decreased during low and high insulin (Figure 5, G and H). High insulin decreased hydroxyproline, whereas EAAs maintained the concentrations at baseline during high insulin (Figure 5I).

## Discussion

We investigated mitochondrial protein synthesis and oxidative enzyme activities with EAA infusion during low or high insulin euglycemic clamps. Mitochondrial protein synthesis, COXIV activity, and whole-body  $VO_2$  increased during EAAs with high insulin concentrations. EAA infusion during low and high insulin concentrations reduced



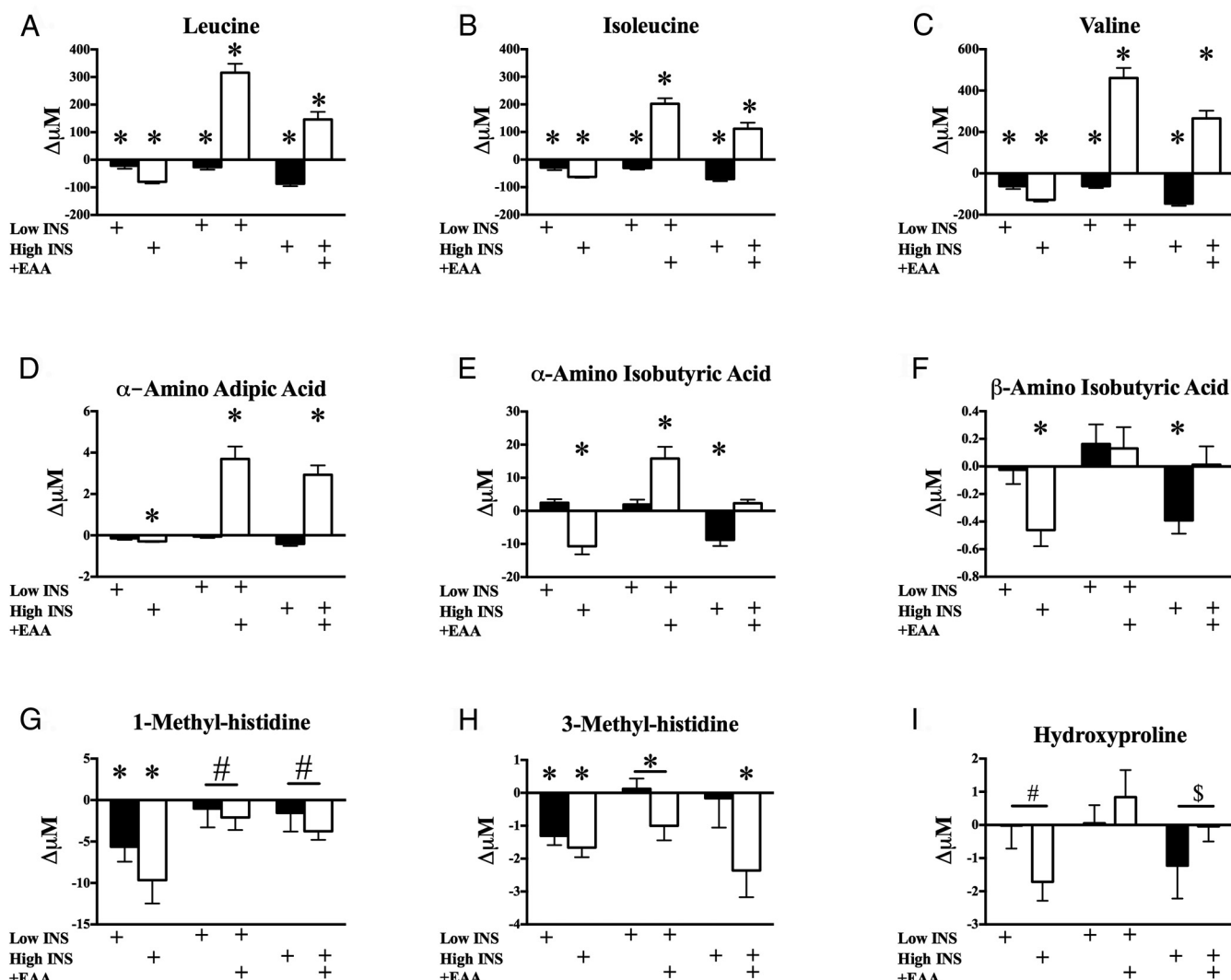
**Figure 4.** Whole-body  $\text{VO}_2$  (A),  $\text{VCO}_2$  (B), and nonprotein RER (C). Carbohydrate (CHO, D) and fat oxidation (E) rates were determined by indirect calorimetry across the whole body. F, Citrate synthase activity was measured from isolated mitochondria and normalized to mitochondrial protein. G, COXIV activity was determined as the maximal enzyme activity normalized to protein content and is expressed as percent increase between study days. Data are means  $\pm$  SEM from  $n = 7$  to 10 per group. \*,  $P < .05$  for paired  $t$  test within each group. #,  $P < .05$  compared with 0. INS, insulin.

whole-body insulin sensitivity based on the GIR needed to maintain euglycemia and increased metabolites related to insulin resistance (eg,  $\alpha$ -amino adipic acid). Furthermore, high insulin suppressed degradation markers for MYO proteins (eg, 3-methylhistidine) and the extracellular matrix (eg, hydroxyproline). These changes in plasma amino acid metabolites during EAAs are consistent with inducing insulin resistance and enhancing the anticatabolic effect of insulin. The complete list of amino acids and metabolites is provided as Supplemental Table 1.

The increase in mitochondrial protein synthesis was more robust during high insulin and EAAs despite an attenuated rise in plasma EAAs. Indeed, EAAs had no dose effect on mitochondrial function or protein synthesis because the greater increase in EAA concentrations during low insulin with EAA infusion showed no increase in COXIV or whole-body  $\text{VO}_2$ . These findings support a hypothesis that mitochondrial protein synthesis is dependent

on high insulin when EAAs are supplemented, but increasing the EAA concentration is not sufficient during low insulin. Our results indicate that higher insulin with sufficient EAAs is necessary to stimulate mitochondrial protein synthesis.

Differential centrifugation yields a sample enriched in mitochondrial proteins that also contains small amounts of nonmitochondrial proteins with varying synthesis rates (1). We used MS and determined that our final pellet was predominantly mitochondrial proteins but included MYO contamination (data not shown). We did not observe a significant effect of high insulin and EAAs on MYO protein synthesis, which is consistent with previous studies showing that the myosin synthesis rate is not enhanced by insulin (22) and insulin and amino acids (13). It is unlikely that contamination by MYO protein contributed to the increased mitochondrial protein synthesis by EAAs and insulin. Additional purification steps are possible but



**Figure 5.** A–C, Branched-chain amino acids during high and low insulin (INS) infusions, with and without EAA infusion. D–F, Plasma  $\alpha$ -amino adipic acid (D),  $\alpha$ - and  $\beta$ -aminoisobutyric acid (E and F) during high and low insulin with and without EAAs. G–I, Methylated histidine metabolites and hydroxyproline during high and low insulin with and without EAAs. \*,  $P < .05$  vs baseline for multiple comparisons after significant two-way ANOVA (study day  $\times$  time) with repeated measures. #,  $P < .05$  for time. \$,  $P < .05$  for study day  $\times$  time interaction.

require larger samples more readily obtained from animal studies (1, 20).

The dependence of mitochondrial protein synthesis on insulin has implications for mitochondrial dysfunction with insulin resistance. Decreased mitochondrial content and function are observed in insulin-resistant humans (23), and previous studies showed that insulin deficiency adversely affected mitochondrial ATP production (24). Yet the relationship between mitochondrial protein turnover and insulin resistance remains to be determined. Chronic elevations in amino acids, particularly branched-chain amino acids, may overwhelm mitochondrial respiratory chain capacity, leading to oxidative stress and impaired glucose uptake (15). We did not measure oxidative stress, yet mouse models support the hypothesis during high-fat feeding (14). Our current study showed that amino acid metabolites increased along with COXIV ac-

tivity and whole-body  $\text{VO}_2$  during EAAs and high insulin consistent with greater mitochondrial respiration. The increased  $\text{VO}_2$  and  $\text{VCO}_2$  with EAAs during high insulin indicates increased mitochondrial respiration, potentially to supply the high ATP demands of protein synthesis (25).

EAA infusion showed a trend to stimulate mixed and fractional protein synthesis rates during low and high insulin concentrations, although a significant change occurred for the mitochondrial fraction during the high insulin and EAA infusions. Such increased protein synthesis with high insulin appears related to extracellular amino acid availability (26). Previous reports indicate that EAA supplementation may attenuate muscle wasting by stimulating MMP synthesis (27, 28). Our data show that EAAs may also benefit muscle protein subfractions, especially the mitochondria, which decline with chronic conditions such as aging (2, 3). Continued increases in mitochondrial

protein synthesis over time may lead to cumulative gains in mitochondrial protein content, yet mitochondrial protein degradation should also be considered. Although our current study was a nutritional intervention, others reported that the types of proteins being synthesized depend on the stimulus, such as resistance or endurance exercise (29). The synthesis rate of myosin heavy chain, which is a key component of MYO proteins, is robustly increased after resistance exercise (30) and may therefore be more responsive to contractile than nutritional stimuli. Additional studies can investigate how specific proteins are preferentially synthesized and degraded to target future therapies toward individual protein subfractions.

Our observation of a reduced GIR required to maintain euglycemia on addition of EAAs to insulin infusion indicates decreased whole-body insulin sensitivity. However, we cannot fully separate the insulin effect on endogenous glucose production and peripheral glucose disposal. Certain amino acids can enhance glucagon secretion, but our study design used a pancreatic clamp that maintained glucagon at basal concentrations, ensuring little to no stimulation of hepatic glucose output by glucagon (31, 32). Previous dose-response curve data from Rizza et al (33) showed that hepatic glucose output is suppressed by 50% with insulin concentrations of  $\sim 28 \mu\text{U/mL}$  and completely suppressed at  $\sim 50 \mu\text{U/mL}$ . Although the above study did not infuse EAAs, the dose response indicates strong suppression of endogenous glucose output and increased glucose disposal at insulin concentrations similar to those in the current study. Of interest, it has been shown that amino acid infusions reduce muscle glucose uptake in humans (34). We also observed that EAAs attenuated the high insulin-induced Akt phosphorylation in skeletal muscle (Supplemental Figure 1), supporting inhibition of insulin signaling in skeletal muscle.

We investigated whether changes in substrate utilization could explain the insulin resistance during EAA infusion. Whole-body substrate oxidation shifted toward carbohydrates during the higher glucose infusion rates with high-dose insulin. However, EAAs did not change the nonprotein RER or whole-body substrate oxidation rates of carbohydrate and fats during low or high insulin. We conclude that the decreased GIR during EAA infusion is not explained by changes to whole-body substrate oxidation. Local changes to amino acid metabolism may have occurred within the muscle but were not detected by indirect calorimetry (16).

High insulin decreased multiple EAAs including branched-chain amino acids and many amino acid metabolites. Increased concentrations of  $\alpha$ -aminoadipic acid, a lysine catabolism product, are predictive of insulin resistance (35). We showed that  $\alpha$ -aminoadipic acid in-

creased with EAA infusion regardless of insulin concentrations, consistent with decreased insulin sensitivity. The metabolite  $\alpha$ -aminoadipic acid is considered a marker of damage to long-lived proteins, such as collagen. Yet, we detected changes over several hours, indicating that concentrations can be regulated acutely, probably released from lysine catabolism. Recently,  $\beta$ -aminoisobutyric acid was identified as a myokine secreted after exercise that stimulates mitochondrial adaptations (36). Accordingly, our results show that mitochondrial protein synthesis and  $\beta$ -aminoisobutyric acid increased during high insulin with EAAs.

Certain posttranslational modifications of amino acids in proteins are only removed by protein degradation and serve as endogenous tracers for specific proteins. MYO protein degradation releases 1- and 3-methylhistidine, commonly measured in urine (37). Plasma 1- and 3-methylhistidine concentrations decreased with low and high insulin infusion, indicating that decreased MYO protein degradation contributed to suppression of whole-body protein degradation. Hydroxyproline is released after extracellular matrix degradation (38) and decreased with high insulin, but was blunted with EAA infusion. The extracellular matrix is considered to have low turnover rates, yet recent investigations reveal that collagen protein synthesis is acutely regulated after exercise (39). Our data indicate that markers for extracellular matrix and MYO protein degradation are decreased by nutritional factors.

In summary, skeletal muscle mitochondrial protein synthesis, oxidative enzyme activity, and whole-body  $\text{VO}_2$  were enhanced by EAAs during high insulin concentrations. High insulin alone, despite higher GIR, showed no increase in skeletal muscle mitochondrial protein synthesis or oxidative enzyme activity, indicating that the insulin effect on mitochondrial function is not driven by increased glucose oxidation. High insulin infusion with EAAs stimulated mitochondrial protein synthesis and activity, whereas low insulin with EAAs did not, despite having higher EAA concentrations. Thus, increasing EAAs alone is unlikely to stimulate mitochondrial protein synthesis or oxidative activity. Moreover, EAAs raised the plasma markers of insulin resistance and decreased insulin sensitivity acutely but enhanced mitochondrial protein synthesis with high insulin. The optimal effect of EAAs on mitochondrial protein synthesis appears to occur when insulin concentrations are also high. We conclude that insulin is an important regulator of mitochondrial protein synthesis during EAA infusion.

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