Amino Acid Availability Regulates the Effect of Hyperinsulinemia on Skin Protein Metabolism in Pigs*

The effects of amino acid supply and insulin infusion on skin protein kinetics (fractional synthesis rate (FSR), fractional breakdown rate (FBR), and net balance (NB)) in pigs were investigated. Four-month-old pigs were divided into four groups as follows: control, insulin (INS), amino acid (AA), and INS + AA groups based on the nutritional and hormonal conditions. L-[ring-13C6]Phenylalanine was infused. FBR was estimated from the enrichment ratio of arterial phenylalanine to intracellular free phenylalanine. Plasma INS was increased (p < 0.05) in the INS and INS + AA groups. Plasma glucose was maintained by infusion of glucose in the groups receiving INS. The interventions did not change the NB of skin protein. However, the interventions affected the FSR and FBR differently. An infusion of INS significantly increased both FSR and FBR, although AA infusion did not. When an AA infusion was added to the infusion of insulin (INS + AA group), FSR and FBR were both lower when compared with the INS group. Our data demonstrate that in anesthetized pigs INS infusion did not exert an anabolic effect, but rather it increased AA cycling into and out of skin protein. Because co-infusion of AAs with INS ameliorated this effect, it is likely that the increased AA cycling during INS infusion was related to AA supply. Although protein kinetics were affected by both INS and AAs, none of the interventions affected the skin protein deposition. Thus, skin protein content is closely regulated under normal circumstances and is not subject to transient changes in AAs or hormonal concentrations.

Skin has multiple functions, most importantly protecting the underlying tissue and organs. The integrity of skin tissue is largely dependent on its protein matrix; thus skin protein metabolism is integral for the maintenance of its function. Previously, our group has developed and successfully implemented a stable isotope method to measure the in vivo fractional synthesis and breakdown rates (FSR and FBR, respectively) of skin protein (1–3). Using this and other methods, we and others have reported the unique features of skin and wound protein metabolism (1, 2, 4) and its responses to nutritional and hormonal interventions (3, 5, 6). Amino acid (AA) supplements and insulin (INS) are of particular interest among these interventional factors due to their well known anabolic effect on muscle and importance in wound healing (7, 8).

AA infusion (Travasol) increased the rates of both skin protein synthesis and breakdown, whereas a combination of INS and AA did not affect either synthesis or breakdown (3). The skin protein NB did not change in response to any treatment as compared with the control group (3). In 7-day-old neonatal pigs, both INS alone and INS + AA increased the skin protein FSR, whereas in 26-day-old pigs only INS + AA increased the skin protein FSR (5). The effect of AA and INS has been also studied in an animal model of skin wounds (9) and donor site wounds in burn patients (10). Zhang et al. (9) showed that in rabbit skin wound, concurrent infusion of AA and INS did not change the rate of synthesis of skin protein, but it significantly decreased the breakdown rate, consequently improving protein net deposition/NB. Sakurai et al. (10) showed that the skin donor site wound protein FSR was not different in patients receiving INS + AA treatment for post-burn glucose control compared with the nontreated group. Careful analysis of the previous studies reveals that for many of these studies (3, 4, 6) INS was administered concurrently with AA, meaning that the effect of the combination of both anabolic agents, INS and AA, was evaluated. Only Davis et al. (5) evaluated the effect of INS alone on skin FSR. Next, for many of these studies only protein

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1 The abbreviations used are: FSR, fractional synthesis rate; FBR, fractional breakdown rate; NB, net balance; CNT, control; INS, insulin; AA, amino acid; BCAA, branched-chain amino acid; EEA, essential AA; NEAA, nonessential AA; TAA, total AA; NB, net balance; PD, priming dose; IR, infusion rate.
synthesis was measured (4–6), which does not necessarily reflect net gain or loss of tissue. Therefore, it appears that the effect of INS and/or AA on skin protein metabolism has not been completely evaluated.

In this study, we aimed to evaluate the effects of INS and AA, either alone or in combination, on skin protein metabolism in pigs by measurement of both FSR and FBR. To measure FBR, a new calculation model, based on the enrichment ratio of arterial stable isotope tracer (l-[ring-13C6]phenylalanine) to skin intracellular free Phe, was designed. Thus, we studied the responses of untreated controls (CNT), AA alone, INS alone, and the combination of INS and AA (INS + AA).

Materials and Methods

Animals
Normal female Yorkshire swine (K-bar Livestock, Sabinal, TX; and John Albert Yorkshire Farm, Cibolo, TX) (weight, 45.4 ± 1.05 kg (mean ± S.D.); age, 3.9 ± 0.1 months) were studied. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (IACUC 00-03-017). The animals were studied. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (IACUC 00-03-017). The animals were studied. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (IACUC 00-03-017). The animals were studied. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (IACUC 00-03-017).

Surgical Procedure

Prior to the start of the experiment, a Swan-Ganz catheter was inserted via the right external jugular vein, and the tip was advanced to the pulmonary artery for infusions of isotope tracer, insulin, and amino acids and for monitoring the body temperature. The body temperature was maintained at ~37 °C throughout the study with the use of a heating blanket. Another catheter was inserted in the carotid artery for arterial blood sampling as well as for blood pressure and heart rate monitoring. An indwelling catheter was placed in the femoral vein for venous blood sampling.

Infusion Protocol

There were four study groups as follows: CNT, INS, AA, and INS + AA. The experimental protocol is illustrated in Fig. 1. After obtaining blood samples to measure background AA enrichment, infusion of l-[ring-13C6]phenylalanine (Cambridge Isotope Laboratories, Andover, MA) was started at time -180 min and was maintained throughout the study (priming dose (PD), 8 μmol/kg; infusion rate (IR), 0.2 μmol/kg.min⁻¹). At 0 min, interventional infusions were started and continued until the end of the study. CNT group was given 0.9% saline; and INS group, insulin (Humulin® R, Lilly) (PD, 20 milliunits/kg; IR, 2.5 milliunits/kg⁻¹ min⁻¹); AA group, AAs (10% Travasol, Baxter Healthcare, Deerfield, IL) (PD, 1.2 ml/kg; IR, 1.8 ml/kg⁻¹ h⁻¹); and INS + AA group, concurrent infusion of insulin and AAs. One hundred ml of Travasol solution contains 730 mg of leucine, 600 mg of isoleucine, 580 mg of lysine, 580 mg of valine, 560 mg of phenylalanine, 480 mg of histidine, 420 mg of theo-

nine, 400 mg of methionine, 180 mg of tryptophan, 2.07 g of alanine, 1.15 g of arginine, 1.03 g of glycine, 680 mg of proline, 500 mg of serine, and 40 mg of tyrosine. Arterial blood glucose concentration was measured every 15 min and was clamped at the basal level by infusion of 20% dextrose solution (Hospira, Lake Forest, IL) in INS and INS + AA groups.

Sample Analyses

Phenylalanine Enrichment—Blood samples for determination of Phe enrichment were immediately precipitated in preweighed tubes containing 15% sulfsalicylic acid. The supernatant was passed over a cation exchange column (Dowex AG 50W-8X, 100–200 mesh H⁺ form; Bio-Rad) and dried under vacuum with a SpeedVac (Savant Instruments, Farmingdale, NY).

Twenty to 25 mg of skin was homogenized twice in 800 μl of 10% perchloric acid. The supernatant and the pellet obtained after tissue homogenization and centrifugation were used for the measurement of the free intracellular and bound Phe enrichment, respectively (11).

Plasma and tissue Phe enrichments were determined after derivatization to tert-butyldimethylsilyl by gas chromatography-mass spectrometry (GC-MS HP 5989; Hewlett-Packard, Palo Alto, CA) with electron impact ionization. Ions 234 and 240 were monitored (11).

Plasma AA Concentration—Plasma samples were analyzed for AA concentrations by high performance liquid chromatography (HPLC System 2690; Waters Alliance, Melford, MA).

Skin Intracellular Free AA Concentration—20–25 mg of skin sample collected at 120 min was homogenized in 3.75% sulfsalicylic acid solution containing 100 nmol/liter of aminoethylecysteine as internal standard, and the intracellular free AA concentrations were measured by HPLC (Hitachi L-8800 amino acid analyzer, Troy, MI).
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Glucose concentration was determined enzymatically by an automated system (YSI 2300; YSI Inc., Yellow Springs, OH). Plasma insulin concentration was determined by a radioimmunoassay method (Linco Research, St. Charles, MO).

Calculations

Area under the curve of insulin during the intervention period was calculated using trapezoid formula.

Ratio of Inward Transport to Intracellular Breakdown (p)

This is calculated from the dilution of the intracellular Phe enrichment relation to the plasma enrichment from Equation 1 (11),

\[ p = \frac{E_M(\text{plateau}) - \Delta E_{\text{plateau}}}{E_P(\text{plateau})} \]  

(Eq. 1)

where \( E_M(\text{plateau}) \) and \( E_P(\text{plateau}) \) are the plateau enrichments in the free intracellular pool and the artery, respectively (11). A derivation of this formula is given under “Appendix.”

FSR

Calculations were based on the precursor-product method, using intracellular free and bound protein tracer enrichments in the skin biopsy as precursor and product, respectively. Equation 2 (11) was used for the calculations,

\[ \text{FSR} = \left( \frac{(E_s - E_{s0})}{(E_f \times t)} \right) \times 60 \times 100 \times 24 \]  

(Eq. 2)

where \( E_f \) and \( E_s \) are the enrichments of free and skin-bound amino acids, respectively, and \( t \) is the time interval in minutes between two sequential biopsies. FSR was expressed in %/day.

FBR

The FBR of skin proteins can be calculated from Equation 3,

\[ \text{FBR} = \frac{(\text{FSR} \cdot \text{CM/CM}0 - \text{QM}'/\text{QT})/(1 + p)} \]  

(Eq. 3)

where FSR is the fractional synthesis rate during the treatment period; FSR0 is the baseline fractional synthesis rate determined before the start of the treatment; \( p \) is the ratio of inward transport to intracellular breakdown at the baseline determined as described above; \( p \) is the ratio of inward transport to intracellular breakdown after treatment; \( QT \) is the amount of phenylalanine in bound protein, assumed to be 20 \( \mu \)mol Phe/g wet skin tissue (2); \( CM \) and \( CM0 \) are the treatment and baseline intracellular skin-free phenylalanine concentrations, and \( QM' \) is the rate that the intracellular skin-free phenylalanine pool size is changing, expressed in units micromoles of Phe/g of wet skin tissue. The assumptions behind the above formula are described below, and the derivation of the formula is presented under the “Appendix.”

Assumption 1—The rate that the free amino acid pool size in skin is changing and equal to the rate that amino acids enter the free amino acid pool (via either inward transport from the circulation or release from bound protein) minus the rate that amino acids exit the free amino acid pool (via either outward transport to the circulation or incorporation into bound protein).

Assumption 2—Under normal conditions, skin protein mass and skin amino acid pool size is maintained; therefore, in the CNT group protein synthesis will be equal to protein breakdown (i.e. FSR = FBR) and inward transport will be equal to outward transport (i.e. FMA = FVM). If either of these equalities do not hold, then there will be changes in either the free or bound amino acid pool sizes.

Assumption 3—The intracellular free amino acid tracer/tracee ratio is not changing at the time of the measurement (i.e. an isotopic steady state holds).

Assumption 4—Outward transport of amino acids from intracellular space is proportional to the intracellular concentration of amino acids.

Assumption 5—Phenylalanine is not synthesized or degraded in skin.

Assumption 6—We assume that under normal conditions there is 20 \( \mu \)mol of Phe/g of wet skin tissue (i.e. wet bound protein (2)).

NB was measured by Equation 4 (11).

\[ \text{NB} = \text{FSR} - \text{FBR} \]  

(Eq. 4)

Statistical Analysis

Values are presented as mean ± S.E., unless otherwise stated. A one-way analysis of variance was used to assess differences in the general parameters as follows: skin protein turnover values and change in AA concentrations. Bonferroni \( t \) test post hoc was subsequently used to assess differences in skin intracellular AA concentrations. A two-way repeated analysis of variance with the factors time and treatment was used to assess differences in the ratio of inward transport to intracellular breakdown between groups and pre- and post-intervention. A \( p \) value of less than 0.05 was considered statistically significant.

Results

There were no significant differences between the groups in body weight, body temperature, mean arterial blood pressure, or plasma glucose concentration during the intervention period (Table 1). Heart rate was significantly (\( p < 0.05 \)) higher in INS and INS + AA groups during the intervention period. Plasma INS concentrations were significantly higher in INS and INS + AA groups (\( p < 0.001 \)) (Table 1).

Plasma, skin, intracellular and bound enrichments of phenylalanine are presented in Table 2. Plasma AA concentrations are presented as a percentage change from the baseline (Fig. 2). Infusion of INS alone significantly (\( p < 0.05 \)) decreased plasma branched-chain amino acid (BCAA) concentration compared with CNT group. Although plasma essential (EAA), nonessential (NEAA), and total AA concentrations also decreased in this group (Fig. 2), the changes did not reach statistical significance. In contrast, infusion of AAs significantly (\( p < 0.001 \) and \( p < 0.05 \)) increased plasma AA concentrations (Fig. 2) compared with CNT and INS groups. In the INS + AA group, changes in BCAA, EAA, NEAA, and total AA concentrations were not significantly different from those in the CNT group but were significantly higher than in INS and lower than in AA groups (\( p < 0.05 \); Fig. 2).
**TABLE 1**

General characteristics of the animals

Data are means ± S.D.; n, number of animals. Values of body temperature (T), heart rate (HR), and mean arterial blood pressure (MAP) are averaged during the intervention period (time 0 to 120 min).

| Group      | Body weight | T    | HR  | MAP | Glucose | Insulin |
|------------|-------------|------|-----|-----|---------|---------|
|            | kg          | °C   | beats/min | mm Hg | mg/dl   | microunits/ml |
| CNT (n = 6) | 49.3 ± 1.4 | 37.7 ± 0.3 | 84 ± 5 | 73 ± 10 | 63 ± 2 | 4.2 ± 0.1 |
| INS (n = 6) | 44.8 ± 0.9 | 37.6 ± 0.8 | 100 ± 5.4 | 76 ± 13 | 69 ± 12 | 150.5 ± 16.6 |
| AA (n = 5)  | 43.3 ± 1.6 | 37.5 ± 0.2 | 91 ± 7 | 81 ± 4 | 61 ± 3 | 4.3 ± 0.7 |
| INS + AA (n = 6) | 44.0 ± 0.3 | 37.0 ± 0.8 | 96 ± 6 | 69 ± 12 | 64 ± 7 | 259.4 ± 33.6 |

* p < 0.05 as compared with the CNT group.
\( ^{a} \) p < 0.001 as compared with the CNT and AA groups.
\( ^{b} \) p < 0.001 as compared with the CNT, AA, and INS groups.

**TABLE 2**

Phenylalanine enrichment data

The tracer enrichments in plasma and skin intracellular and bound pools at 30 min before (Pre) and 120 min after (Post) the intervention in CNT, INS, AA, and INS + AA groups. Data are presented as tracer to tracer ratio, means ± S.D. Statistical analyses were performed using two-way analysis of variance repeated measures; p < 0.05 was considered statistically significant.

| Groups | Plasma | Intracellular | Bound |
|--------|--------|---------------|-------|
|        | Pre    | Post          | Pre   | Post          |
| CNT    | 0.146 ± 0.045 | 0.146 ± 0.035 | 0.054 ± 0.017 | 0.064 ± 0.021 |
| INS    | 0.149 ± 0.032 | 0.174 ± 0.036 | 0.044 ± 0.015 | 0.052 ± 0.015 |
| AA     | 0.125 ± 0.016 | 0.106 ± 0.006 | 0.057 ± 0.014 | 0.068 ± 0.010 |
| INS + AA | 0.138 ± 0.030 | 0.102 ± 0.019 | 0.053 ± 0.015 | 0.069 ± 0.019 |

* Data are statistically different between the periods.
\( ^{a} \) Data are statistically different compared with the CNT group within the period.
\( ^{b} \) Data are statistically different compared with the INS group within the period.

**FIGURE 2.** Percentage change of the plasma BCAA, EAA, NEAA, and TAA concentrations relative to the basal levels. Data are presented as means ± S.E. \( ^{a} \) p < 0.05 versus CNT group; \( ^{b} \) p < 0.001 versus CNT; \( ^{c} \) p < 0.05 versus INS group; \( ^{d} \) p < 0.001 versus INS; \( ^{e} \) p < 0.05 versus AA group.

Skin intracellular AA concentration data are presented in Table 3. The most significant change was that an infusion of INS completely diminished Pro in all samples. Although INS infusion did not decrease the concentrations of individual BCAAs, the sum of BCAAs was significantly \( (p < 0.05) \) decreased in this group when compared with the CNT group. In AA and INS + AA groups, there were no significant changes, except a decrease in glutamate (Glx) in the AA group compared with CNT group and an increase in asparagine (Asx) in INS + AA group compared with AA group (Table 3).

The ratio of inward transport to the breakdown in the basal period was not different between the groups (Fig. 3). INS infusion did not change the ratio \( (p > 0.05) \). INS + AA infusion significantly increased the ratio of inward transport to the breakdown \( (p < 0.001) \) compared with the basal level and compared with CNT, INS, and AA \( (p < 0.001 \) or \( p < 0.05) \) groups during the intervention period.

Skin protein FSR, FBR, and NB are presented in Fig. 4. INS infusion increased skin FSR 2–3-fold compared with the CNT, AA, and INS + AA groups \( (p < 0.05) \). The average FSRs in AA and INS + AA groups were 40 and 50% higher than in the CNT group; however, the difference did not reach statistical significance \( (p > 0.05) \). INS infusion significantly increased skin FBR when compared with the CNT group \( (p < 0.05) \). It was also significantly higher than in INS + AA \( (p < 0.05) \) but not AA \( (p = 0.11) \). In AA and INS + AA groups, FBR was 20% lower and higher than in the CNT group, respectively. However, the differences were not statistically significant.

**TABLE 3**

Skin intracellular amino acid composition

Comparison of skin samples collected at 120 min is shown. Data are means ± S.D. in μmol/liter tissue water content. Ax indicates sum of asparagines and aspartic acid; Glx indicates sum of glutamine and glutamic acid; TAA indicates total amino acids.

| Amino acids | CNT | INS | AA | INS + AA |
|-------------|-----|-----|----|---------|
| Asx         | 324 ± 69 | 281 ± 60 | 217 ± 110 | 365 ± 83 |
| Glx         | 2459 ± 608 | 1914 ± 390 | 1033 ± 258 | 1879 ± 342 |
| Ser         | 1567 ± 681 | 1131 ± 239 | 1215 ± 935 | 1175 ± 323 |
| His         | 483 ± 259 | 326 ± 63 | 406 ± 330 | 393 ± 105 |
| Tyr         | 86 ± 52 | 62 ± 67 | 50 ± 55 | 45 ± 62 |
| Pro         | 309 ± 176 | 0 ± 0 | 464 ± 195 | 374 ± 88 |
| Thr         | 472 ± 168 | 309 ± 83 | 406 ± 197 | 385 ± 59 |
| Val         | 326 ± 89 | 210 ± 36 | 291 ± 145 | 311 ± 68 |
| Met         | 23 ± 36 | 5 ± 12 & | 48 ± 16 | 58 ± 39 |
| Phe         | 181 ± 64 | 185 ± 43 | 138 ± 54 | 198 ± 27 |
| Ile         | 88 ± 52 | 25 ± 39 & | 145 ± 72 | 154 ± 91 |
| Leu         | 424 ± 118 | 258 ± 63 | 276 ± 154 | 364 ± 131 |
| Lys         | 270 ± 57 | 150 ± 44 | 309 ± 139 | 240 ± 85 |
| BCAA        | 783 ± 206 | 434 ± 86 | 730 ± 300 | 760 ± 252 |
| EAA         | 2272 ± 749 | 1471 ± 180 | 2029 ± 993 | 2107 ± 433 |
| NEAA        | 9036 ± 2441 | 6388 ± 847 | 6694 ± 3227 | 7595 ± 1653 |
| TAA         | 11308 ± 3150 | 7856 ± 998 | 8723 ± 4206 | 9702 ± 1970 |

* p < 0.05 as compared with the AA group.
\( ^{a} \) p < 0.05 as compared with the INS + AA group.
\( ^{b} \) p < 0.001 as compared with the CNT group.
\( ^{c} \) p < 0.001 as compared with the INS + AA group.
\( ^{d} \) p < 0.05 as compared with the CNT group.
Insulin Increases Skin Protein Synthesis and Breakdown Rates

**Discussion**

The main finding of this study is that the availability of AAs modulated the effect of hyperinsulinemia on skin protein metabolism. The data demonstrate the complexity of the effect of AA and INS on skin protein metabolism in pigs and suggest that evaluation of both the synthesis and breakdown rates of skin protein is needed to understand the effect of interventions on skin protein metabolism.

**Effect of Hyperinsulinemia on Skin Protein Metabolism**—Insulin plays an important role in the regulation of protein and AA metabolism. Our results show that INS significantly increased skin protein FSR in 3.9-month-old pigs (Fig. 4), which would appear to be at odds with the findings of Davis et al. (5), where they reported that insulin significantly increased FSR in 7-day-old but not in 26-day-old pigs. However, this discrepancy can be explained by the difference in the plasma INS concentration (150 versus 27.3 microunits/ml, this study versus Davis et al. (5)). Another study of Davis et al. (4) demonstrated that skin protein FSR varies depending on plasma INS concentration. The novelty of our work is that we measured both FSR and FBR, which enabled us to estimate skin protein NB. Our results showed that in the INS group the FBR also increased significantly (Fig. 4). This result is consistent with previous conclusions that in muscle the effect of INS on protein metabolism depends on AA availability (12–18). Protein synthesis requires sufficient AA supply. AAs can be supplied by transport from artery into the cell or by breakdown of intracellular proteins. The ratio of inward transport to intracellular breakdown (defined as $p$) gives a value characterizing the relative contribution from the two pathways. In the INS group, this ratio of inward transport to breakdown did not change compared with the CNT group (Fig. 3); this can be interpreted as either INS did not affect either of these pathways or that INS affected both pathways to the same extent. In the INS group, FSR increased by 0.35%/h. If we assume that there is 20 $\mu$mol of Phe/g of wet skin tissue, then this is an increase of 0.35%/h $\times$ 20 $\mu$mol of Phe/g of wet tissue = 0.07 $\mu$mol of Phe/g of wet tissue/h. If transport and breakdown did not change over the 2 h that we gave INS, then the free intracellular Phe concentration should have dropped by 0.07 $\mu$mol of Phe/g of wet tissue/h $\times$ 2 h = 0.14 $\mu$mol of Phe/g of wet tissue. We found a Phe concentration of 0.185 $\mu$mol of Phe/ml of tissue water in INS versus 0.181 in the CNT group (Table 3), so the breakdown rate must have increased to achieve the total increase of 0.18 $\mu$mol of Phe/g of wet tissue/2 h (0.14 + 0.04 = 0.18 $\mu$mol of Phe/g of wet tissue/2 h = 0.09 $\mu$mol of Phe/g of wet tissue/h) in the INS group. Therefore, because the $p$ value did not change during INS infusion, both inward transport and protein breakdown should have been affected to maintain the measured AA concentration. Apparently, how INS affects skin protein metabolism (FSR and FBR) differs depending on AA availability (Fig. 4). When only INS was given, plasma AA concentration significantly dropped (Fig. 2); therefore, even though inward transport increased, the supply of AA was not enough to match the intracellular AA demand for a 3-fold increase in FSR (Fig. 4). We observed a similar situation in the case of muscle protein synthesis in burned children (10). In the latter case, muscle protein breakdown increased significantly during insulin infusion because inward transport of AAs was insufficient to maintain intracellular availability of amino acids in the context of stimulated synthesis. This caused an increase in breakdown, which served to maintain intracellular AA levels to at least some extent (10). Interestingly, the NB in the INS group did not differ significantly when compared with the CNT group (Fig. 4). This demonstrates that under these study conditions, the INS infusion did not increase the net balance of protein in the skin but stimulated amino acid cycling. Interestingly, we also observed an increased heart rate in this group of pigs (Table 1), which may support our notion of increased substrate cycling in these animals.

**Effect of AA and INS + AA on Skin Protein Metabolism**—The infusion of AA did not affect any of the measured parameters, e.g. FSR, FBR, and NB (Fig. 4), inward transport (Fig. 3), and intracellular AA content (Table 3), except of plasma concentration of AAs (Fig. 2). This may suggest that AAs by themselves affected neither cellular AA transport systems nor the mechanisms of skin intracellular protein metabolism. However, when hyperinsulinemia-induced hypoaminoacidemia was reversed by infusion of exogenous AAs (i.e. INS + AA group), the inward transport of AAs into the skin cells was significantly increased compared with all other groups (Fig. 2). Interestingly, the change in FSR during AA infusion was not significantly different ($p > 0.5$) from the CNT group, but it was significantly lower when compared with the INS group ($p = 0.003$; Fig. 4). At the same time FBR was significantly lower than in the INS group only ($p = 0.020$; Fig. 4). These data suggest that INS is capable of stimulating inward transport of AAs, which was sufficient to
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maintain intracellular AA concentrations (Table 3). Because INS normally suppresses protein breakdown (19), the increase in protein breakdown that occurred during INS infusion alone was somewhat surprising. However, the reversal of hyperinsulinemia-induced increased breakdown by AA infusion may indicate that the fall in intracellular AA concentrations during INS infusion alone was responsible for the increased rate of breakdown. The changes in synthesis and breakdown in AA and INS + AA groups differed from our previous work on rabbit skin protein metabolism (3); however, this can be explained by the fact that in this study we measured FSR and FBR, but in Zhang et al. (3) we used a three-pool model, which represents not only the tissue of interest, i.e. skin, but also muscle. Nevertheless, in this study the NB did not change in any of the intervention groups, which supports our previous reports from Zhang et al. (3) that skin protein mass is maintained in all conditions.

The changes of skin-free AA concentrations were striking. First, the infusion of INS alone completely depleted intracellular Pro (Table 3). Pro composes about 1/6 of collagen AA sequence, the main structural protein of dermis. Considering that skin protein FSR increased 3-fold with INS infusion, we speculate that complete depletion of Pro can be explained by utilization of all available Pro for protein synthesis. However, it is unclear why other AAs did not decrease to the same extent, particularly because proline can usually be produced in the body at a rate sufficient to meet metabolic requirements. The relatively selective depletion of proline can be explained by its pool size and/or its percentage composition of skin protein, particularly collagen. For example, Gly is another AA that is even more abundant in collagen; it composes 1/3 of collagen sequence. The intracellular concentration of Gly decreased by about 25% during INS infusion (Table 3). Considering the abundance of Gly in collagen AA sequence, it would be logical to think that it would have decreased at least to the same extent as Pro. However, from the actual intracellular content of these AAs (Table 3), we can see that there is 7.5-fold difference between Gly and Pro intracellular content, with Gly being much more abundant; therefore, pool size of the Gly is much higher, and this may explain the nonsignificant decrease in Gly concentration. Second, the sum of BCAA was significantly decreased in the INS group compared with the CNT group (Table 3). It is interesting because BCAA are known to stimulate protein FSR, but this was not the case in this study. We observed that skin protein FSR increased despite a decrease in the BCAAs. Although we do not have an explanation for this phenomenon, one possibility is that decreased BCAA availability stimulated protein breakdown, thereby increasing intracellular AA supply.

To calculate FBR, we have designed a new model that builds on six basic assumptions. 1) The rate that the free amino acid pool size in skin is changing is equal to the rate that amino acids enter the free amino acid pool minus the rate that amino acids exit the free amino acid pool. 2) In normal conditions, skin protein mass and skin amino acid pool size are maintained. 3) An isotopic steady state exists. 4) Outward transport of amino acids from the intracellular space is proportional to the intracellular concentration of amino acids. 5) Phenylalanine is not synthesized or degraded in skin. 6) Under normal conditions, there is 20 μmol of Phe/g of wet skin tissue. Each of these assumptions will be discussed. The first assumption is based on the Fick principle. If this assumption was not true, then amino acids would either accumulate or abate in one or more of the compartments. We assume that the Fick principle also holds for labeled amino acids (tracers). The second assumption is based on our previous reports that in normal conditions, skin protein mass and skin amino acid pool size are maintained (3). The third assumption can be assessed directly by observing the change in intracellular free amino acid tracer/tracee ratio over time or indirectly by observing the change in plasma amino acid tracer/tracee ratio over time. To increase the likelihood of an isotopic steady state, we minimized changes in amino acid tracer/tracee ratio in this study by adding an appropriate amount of labeled amino acids to the Travasol mixture. Next, there was no significant difference in plasma phenylalanine enrichment between 90 and 120 min of each study period, which confirms an isotopic steady state of phenylalanine. Furthermore, we and others have shown on numerous occasions that an isotopic steady state of phenylalanine is achieved after 120 min of infusion. The fourth assumption is difficult to test directly, but the intracellular phenylalanine concentration did not change much in this study for any of the treatments. The fifth assumption is based on the fact that phenylalanine is an essential amino acid, so one would not expect it to be synthesized. Assumption 6 is based on previous results (2).

The results of this study demonstrate the complexity of the effect of hyperinsulinemia on in vivo substrate kinetics in skin. These results may have a potentially significant translational value. Wound healing is a dynamic and complex biological process that can be divided into four partly overlapping phases as follows: hemostasis, inflammation, proliferation, and remodeling. Insulin injection is used to treat diabetes mellitus and also to control burn injury-related hyperglycemia. Thus, with a proper supply of AA, the treatment-related hyperinsulinemia may have a potentially beneficial effect on skin wound healing (i.e. proliferation).

However, there are some limitations in our study. We did not evaluate the molecular mechanisms of the effect of AAs and/or hyperinsulinemia on insulin signaling as well as other regulatory pathways for protein metabolism, e.g. eukaryotic initiation factors, which are known to be pivotal in controlling protein metabolism. Thus, future studies to pursue this topic and to evaluate the molecular mechanisms along with the substrate kinetics are warranted.

In conclusion, the data, generated under the experimental conditions of this study, support our previous findings that skin protein mass is maintained under different conditions (3). These data also demonstrate that the substrate (e.g. AA) fluxes in skin cells differ depending on the substrate availability and that INS may control the mechanism of protein synthesis but not the breakdown. Also, to fully understand the effect of different interventions on skin protein metabolism, it would be beneficial to measure both the synthesis and breakdown rates. Future studies evaluating its relevance in clinical conditions, e.g. diabetic foot ulceration and long term insulin treatments, would be of interest.
Insulin Increases Skin Protein Synthesis and Breakdown Rates

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Appendix

We will assume that the intracellular free amino acid pool acts like a single pool and that the intracellular free amino acid pool size (denoted \( Q_M(t) \)) is changing at rate \( Q_M(t) \). We will assume that the rate that the intracellular free amino acid pool size is changing is equal to the rate that unlabeled amino acids enter the intracellular free pool via protein breakdown (denoted \( PB \)) and inward transport (denoted \( F_{V,M} \)) minus the rate that unlabeled amino acids exit the intracellular free pool via protein synthesis (denoted \( PS \)) and outward transport (denoted \( F_{V,M} \)), as expressed by Equation 5,

\[
Q_M(t) = PB(t) + F_{V,M}(t) - PS(t) - F_{V,M}(t) \quad \text{(Eq. 5)}
\]

We assume here that there is no other source of AAs.

Equation 5 can be written as shown in Equation 6,

\[
PS(t) + F_{V,M}(t) = PB(t) + F_{V,M}(t) - Q_M(t) \quad \text{(Eq. 6)}
\]

We will assume that tracer only enters the intracellular space from blood. Under most circumstances, the enrichment of bound protein will be a thousand-fold lower than the blood enrichment, so this is a reasonable assumption. The rate that tracer enters into the intracellular space at a given time \( t \) will thus be equal to the rate that the tracer is transported into the cell times the tracer to tracer ratio of blood amino acids, i.e. \( E_A(t)F_{M,A}(t) \). Likewise, the rate that the tracer leaves the intracellular space (via both transport and incorporation into protein) at a given time \( t \) will be equal to the rate that the tracer leaves the intracellular space times the tracer to tracer ratio of intracellular amino acids, i.e. \( E_M(t) \).

The rate that the intracellular tracer pool size is changing (denoted \( q_M(t) \)) will be equal to the rate that tracer enters the intracellular pool minus the rate that tracer leaves the intracellular pool shown in Equation 7,

\[
q_M(t) = E_A(t) \cdot F_{M,A} - E_M(t) \cdot (PS(t) + F_{V,M}(t)) \quad \text{(Eq. 7)}
\]

Now because the intracellular enrichment is by definition equal to the ratio of the tracer to tracer ratio of intracellular pool sizes (i.e. \( E_M(t) = q_M(t)/Q_M(t) \)), we can conclude the following Equation 8,

\[
q_M(t) = E_A(t) \cdot Q_M(t) \quad \text{(Eq. 8)}
\]

Differentiating both sides of Equation 8 with respect to time gives Equation 9,

\[
q_M(t) = E_A(t) \cdot Q_M(t) + E_M(t) \cdot Q_M'(t) \quad \text{(Eq. 9)}
\]

So combining Equations 7 and 9 gives Equation 10,

\[
E_A(t) \cdot Q_M + E_M(t) \cdot Q_M(t) = E_A(t) \cdot F_{M,A}(t) - E_M(t) \cdot (PS(t) + F_{V,M}(t)) \quad \text{(Eq. 10)}
\]

If we substitute Equation 6 into Equation 10, then we get Equation 11,

\[
E_A(t) \cdot Q_M(t) + E_M(t) \cdot Q_M(t) = E_A(t) \cdot F_{M,A}(t) - E_M(t) \cdot (F_{V,M}(t) + PB(t) - Q_M(t)) \quad \text{(Eq. 11)}
\]

We will define the variable \( p(t) \) by Equation 12,

\[
p(t) = F_{V,M}(t)/PB(t) \quad \text{(Eq. 12)}
\]

If we assume that \( E_A(t) = 0 \), then Equation 11 can be simplified to Equation 13,

\[
p(t) = F_{V,M}(t)/PB(t) = E_M(t)/(E_A(t) - E_M(t)) \quad \text{(Eq. 13)}
\]

Therefore \( p(t) \) can be calculated directly from raw data, and rearranging Equation 12 gives Equation 14,

\[
F_{V,M}(t) = p(t) \cdot PB(t) \quad \text{(Eq. 14)}
\]

At baseline (defined as \( t = 0 \)), we will assume that the bound and free pools in the skin are not changing, and as a result \( PB(0) = PS(0) \) and \( F_{V,M}(0) = F_{M,A}(0) = p(0)PB(0) \). Therefore, once synthesis is known at baseline, all other parameters at baseline can be determined.

We will further assume that outward transport is proportional to the intracellular free amino acid concentration (denoted as \( C_{M,M}(t) \)), i.e. Equation 15,

\[
F_{V,M}(t)/F_{V,M}(0) = C_M(t)/C_M(0) \quad \text{(Eq. 15)}
\]

which can be expressed as Equation 16,

\[
F_{V,M}(t) = F_{V,M}(0) \cdot C_M(t)/C_M(0) \quad \text{(Eq. 16)}
\]

Substituting Equations 14 and 16 into Equation 5 gives Equation 17,

\[
Q_M(t) = (1 + p(t)) \cdot PB(t) - PS(t) - F_{V,M}(0) \cdot C_M(t)/C_M(0) \quad \text{(Eq. 17)}
\]

If we divide both sides of Equation 17 by the bound amino acid pool size (denoted \( Q_f \)), we get Equation 18,

\[
Q_M(t)/Q_f = (1 + p(t)) \cdot PB(t)/Q_f - PS(t)/Q_f - F_{V,M}(0) \cdot C_M(t)/C_M(0) \quad \text{(Eq. 18)}
\]

Because by definition \( FSR = PS(t)/Q_f(t) \) and \( FBR = PB(t)/Q_f(t) \), Equation 18 becomes Equation 19,

\[
Q_M(t)/Q_f = (1 + p(t)) \cdot FBR(t) - FSR(t) - F_{V,M}(0) \cdot C_M(t)/C_M(0) \quad \text{(Eq. 19)}
\]

Solving Equation 19 for \( FBR(t) \) gives Equation 20,

\[
FBR(t) = (FSR(t) + F_{V,M}(0)/Q_f \cdot C_M(t)/C_M(0)) + Q_M(t)/Q_f(1 + p(t)) \quad \text{(Eq. 20)}
\]
As noted above, \( F_{\text{V.M}}(0)/Q_T(0) = p(0) \cdot PB(0)/Q_T(0) = p(0) \cdot PS(0)/Q_T(0) = p(0) \cdot FSR(0) \), so we get Equation 21,

\[
\text{FBRT}(t) = (FSR(t) + p(0) \cdot FSR(0) \cdot C_M(t)/C_M(0)) + Q_M(t)/Q_T(t)/(1 + p(t)) \quad \text{(Eq. 21)}
\]

which is the desired equation.

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