Anabolic action of insulin on skin wound protein is augmented by exogenous amino acids

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Zhang, Xiao-Jun, David L. Chinkes, Øivind Irtun, and Robert R. Wolfe. Anabolic action of insulin on skin wound protein is augmented by exogenous amino acids. Am J Physiol Endocrinol Metab 282: E1308–E1315, 2002; 10.1152/ajpendo.00361.2001.—To investigate the metabolic basis of skin wound healing, we measured in anesthetized rabbits the responses of protein kinetics in scalded skin to insulin and amino acids. L-[ring-13C6]Phe was infused on the 7th day after the ear was scalded, and the scalded ear was used as an arteriovenous unit to reflect protein kinetics in skin wound. The ipsilateral carotid artery was clamped to control the wound blood flow within four- to fivefold the normal skin rate to measure the enrichment difference in the scalded ear during hyperaminoacidemia. Neither insulin (2.5 mU·kg⁻¹·min⁻¹) nor amino acid (2.5 mg·kg⁻¹·min⁻¹) infusion alone improved net protein balance in skin wound. In contrast, combined infusion of insulin and amino acids increased the net protein balance in skin wound from −6.5 ± 4.5 to 1.4 ± 5.2 μmol·100 g⁻¹·h⁻¹ (P < 0.01, control vs. insulin plus amino acids). We conclude that there is an interactive effect of insulin and sufficient amino acid supply on protein metabolism in skin wound, meaning that their combined anabolic effect is greater than the sum of their individual effects.

stable isotopes; gas chromatograph-mass spectrometer; rabbit ear; arteriovenous balance

INSULIN IS A KEY ANABOLIC HORMONE and not only plays an important role in substrate metabolism but also is a regulator of protein synthesis and breakdown. The effect of insulin on muscle protein has been investigated extensively in the normal state (2, 6, 7, 9, 11–13, 18, 21) and also in catabolic states such as after severe burns (5, 17). However, although the general role of insulin in wound healing is well known (10, 14, 16), the effect of insulin on protein metabolism in skin wound has not been assessed sufficiently.

The lack of information regarding the effect of insulin on protein metabolism in skin wound is largely because of the lack of a method to quantitate both protein synthesis and breakdown in vivo. To this end, we developed a rabbit ear model for measurement of protein metabolism in skin wound (24, 26). Using this animal model, we demonstrated in our previous experiment (24) that insulin had an anabolic effect on wound protein resulting from the inhibition of protein breakdown. It is possible that the failure of insulin to stimulate protein synthesis was because of an insufficient supply of amino acids (AAs). Although AAs were infused during the insulin infusion, the plasma AA concentrations were only maintained at levels that were either comparable to the postabsorptive state (during insulin infusion at 0.6 mU·kg⁻¹·min⁻¹) or even lower (during insulin infusion at 2.3–3.4 mU·kg⁻¹·min⁻¹). Consequently, the potential role of sufficient AA supply in conjunction with insulin on wound protein metabolism is not clear.

The present study was designed to investigate the potential interactive or additive effect of insulin and abundant AA supply on protein metabolism in skin wound. We selected a dose of insulin of 2.5 mU·kg⁻¹·min⁻¹, which is comparable to the higher-dose insulin (2.3–3.4 mU·kg⁻¹·min⁻¹) in a previous experiment (24). We used the same AA solution (i.e., 10% Trasvasol) that we used in the previous experiment, but at a higher rate of infusion (2.5 mg·kg⁻¹·min⁻¹). This dose of AA infusion delivered AA nitrogen at a rate close to the average rate of oral nitrogen intake of normal rabbits and was known from pilot studies to increase plasma AA concentrations to more than two times the postabsorptive levels.

METHODS

Animals. Male New Zealand White rabbits (Myrtle’s Rabbits, Tompsoon Station, TN), weighing ~4.5 kg, were used for this study. The rabbits were housed in individual cages and were given 150 g of Lab Rabbit Chow HF 5326 (Purina Mills, St. Louis, MO) per day for weight maintenance. This feeding regime provided 4.7 g of crude protein and 38 kcal·kg⁻¹·day⁻¹. This study was approved by the Animal Care and Use Committee of The University of Texas Medical Branch at Galveston.

Isotopes. L-[ring-13C6]Phe (99% enriched), L-[ring-2H5]Phe (98% enriched), and L-[2H7]proline (97–98% enriched) were purchased from Cambridge Isotope Laboratories (Woburn, MA). L-[ring-13C6]Phe was used as the tracer for intravenous infusion. L-[ring-2H5]Phe and L-[2H7]proline were used as
internal standards for measurement of Phe and proline concentrations in blood and in the free AA pool in skin wound. Partial-thickness skin wound. A partial-thickness thermal injury was created on the ear skin by submerging the ear in 72°C water for 3 s, as we described in the previous publications (24, 26). Immediately after the scald, a single dose of antibiotic (Bicillin; 50,000 U/kg; Wyeth Laboratories, Philadelphia, PA) was injected intramuscularly. When the animals had awakened from anesthesia, they were given intramuscular injections of buprenorphine (0.03 mg/kg) two times a day for 3 days as an analgesic.

Control of wound blood flow rate. Our previous data showed that on the 7th day after injury the rate of blood flow in the scalded skin was 9- to 15-fold greater than that in the normal skin, and the arteriovenous (a-v) difference of tracer enrichment was only 3–6% of the arterial enrichment (24). In a pilot study, we found that the infusion of a large dose of AAAs caused the a-v difference to become too small to reliably measure. Consequently, we reduced the wound blood flow rate to within four- to fivefold the normal skin rate by clamping the ipsilateral carotid artery in the present study. Our previous experiments showed that the rates of protein synthesis in normal skin and 7-day scalded skin were 12.8 ± 2.9 and 26.6 ± 8.7 μmol·100 g⁻¹·h⁻¹, respectively (24, 26). We considered that a four- to fivefold increase in wound blood flow should be sufficient to supply nutrients for a twofold increase in synthesis. The clamping procedure increased the a-v difference of tracer enrichment, thereby enabling measurement of protein and AA kinetics in the skin wound under the hyperaminoacidemic condition.

Experimental design. There were five groups as follows: control group (n = 10), insulin group (n = 8), AA group (n = 7), insulin-AA group (n = 7), and microsphere group (n = 4). The scald injury was created on the left ear in the control, insulin, AA, and insulin-AA groups and on the right ear in the microsphere group. After the scald injury, the rabbits were given the same amount of food as before the injury, and daily food consumption was recorded. The food was removed at 1700 on the 6th day, and water was available all the time. After an overnight fast, the isotope infusion or microsphere injection studies started at 0800 on the 7th day. The rabbits, except in the microsphere group, received L-[ring-¹³C₆]Phe infusion under different regimens of acute AAAs and/or insulin infusion. In the control group, neither insulin nor AAAs were infused. In the insulin and AA groups, either insulin (Humulin; Eli Lilly, Indianapolis, IN) or AAAs (10% Travasol; Baxter Healthcare, Deerfield, IL) were infused. In the insulin-AA group, both insulin and AAAs (i.e., 10% Travasol) were infused. Insulin was infused at 2.5 μU·kg⁻¹·min⁻¹ in 0.5% albumin solution (10 ml/h), and the Travasol solution was infused at 2.5 mg·kg⁻¹·min⁻¹ (1.5 ml·kg⁻¹·h⁻¹). One hundred milliliters of the Travasol solution contain 730 mg leucine, 600 mg isoleucine, 580 mg lysine hydrochloride, 580 mg valine, 560 mg phenylalanine, 480 mg histidine, 420 mg threonine, 400 mg methionine, 180 mg tryptophan, 2.07 g alanine, 1.15 g arginine, 1.03 g glycine, 680 mg proline, 500 mg serine, and 40 mg tyrosine.

The anesthetic and surgical procedures were described in our previous publications (23–27). In brief, the rabbits were anesthetized with ketamine and xylazine. Polyethylene catheters (PE-90; Becton-Dickinson, Parsippany, NJ) were inserted in the right femoral artery and vein through an incision on the groin. The arterial line was used for blood collection and monitoring of heart rate and mean arterial blood pressure; the venous line was used for infusion. A tracheal tube was placed via tracheotomy. The free end of the tracheal tube was placed in an open hood that was connected to an oxygen supply line so that the rabbits spontaneously breathed oxygen-rich room air.

The central artery of the scalded ear was isolated for placement of a flow probe (model 1RB; Transonic Systems, Ithaca, NY). The flow probe was connected to a small animal blood flowmeter (model T106; Transonic Systems) for measurement of blood flow rate. The ipsilateral carotid artery was isolated, and a bulldog clamp was placed on the artery to control the blood flow rate in the scalded skin.

In the microsphere group, additional procedures were performed to insert a 4.0-Fr polyurethane catheter (Cook Critical Care, Bloomington, IN) in the left ventricle via the left carotid artery to inject microspheres (26). Heparin (50 U/kg) was injected intravenously to prevent coagulation in the catheter placed in the left ventricle.

The isotope infusion protocol is shown in Fig. 1. After completion of the surgical procedures, a blood sample was taken from the arterial line, and a skin sample was taken from the groin incision for measurement of background enrichment. The infusion of L-[ring-¹³C₆]Phe was started after collection of the background samples in the control group. In the insulin, AA, and insulin-AA groups, the infusion of insulin (prime: 20 μU/kg; infusion rate: 2.5 μU·kg⁻¹·min⁻¹) and/or Travasol (prime: 100 mg/kg; infusion rate: 2.5 mg·kg⁻¹·min⁻¹) was started 1 h before the start of the tracer infusion. The dose of L-[ring-¹³C₆]Phe was 0.12 μmol·kg⁻¹·min⁻¹ (prime: 4.8 μmol/ml) in the insulin group and 0.15 μmol·kg⁻¹·min⁻¹ (prime: 6.0 μmol/ml) in the other three groups. In the insulin and insulin-AA groups, a 25% glucose solution was infused at various rates to maintain euglycemia. Arterial glucose concentration was measured every 10 min to estimate the rate of glucose replacement. Throughout the experimental period, the position of the bulldog clamp was adjusted to either totally or partially block the carotid artery to control the wound blood flow rate within four- to fivefold the normal skin rate.

During the 150–240 min of the tracer infusion, four paired arterial and ear-venous blood samples were drawn at intervals of ~20 min. The arterial blood was collected from the femoral artery catheter, and the venous blood was obtained by directly puncturing the marginal ear vein. The blood flow rate in the ear was recorded from the blood flowmeter at each a-v blood sampling. At 240 min, a skin specimen was taken from the ventral side of the scalded ear. The blood samples were kept in an ice-water bath until the end of the infusion study. The skin samples were immediately frozen in liquid nitrogen and stored at ~80°C for later analysis. Additional blood was taken for measurement of plasma concentrations of insulin and AAAs. At the end of the experiment, both ears were cut off at the skin fold between the base and auricle to measure the ear weight.

In the microsphere group, the blood flow rate in the scalded skin was recorded every 10 min in the first 40–60
min to obtain a baseline value. When the blood flow rate reached a constant value, the measurement of capillary flow was performed by injection of microspheres into the left ventricle. The injections were performed when the ipsilateral carotid artery was either clamped or uncloamped in a random order. During the clamp periods, the blood flow rate in the right scalded ear was reduced to four- to fivefold the normal skin rate by adjusting the position of a bulldog clamp that was placed on the ipsilateral carotid artery.

The colored dye-extraction microspheres (15 μM DIA Fluorescent Microspheres; Interactive Medical Technology, Los Angeles, CA) were injected into the left ventricle to measure capillary flow in the skin wound. The injection procedure was described in detail in our previous publication (26). Each injection contained 1.25 × 10^6 spheres of randomized colors. Each rabbit received three injections of microspheres, which has been reported to be acceptable in the rabbit (4). There was an interval of 15 min between injections. The rate of blood flow in the scalded ear was recorded from the blood flowmeter at the time of each microsphere injection. The animals were killed by intravenous injection of 5 ml of saturated KCl under anesthesia. Skin samples were taken from the scalded ears. Both kidneys were taken via laparotomy to ascertain if the injected microspheres were uniformly distributed in the blood. The tissues were kept in individual tubes, and the tissue weight was recorded. The samples were shipped to Interactive Medical Technology for analysis.

Heart rate, mean arterial blood pressure, and rectal temperature were maintained stable by adjusting the infusion rates of anesthetics and physiological saline and by use of heating lamps. These vital signs were continuously monitored throughout the experiment and recorded every 30 min. The surface temperature of the scalded skin was maintained at 37°C by means of a heating lamp.

**Sample analysis.** After completion of the isotope infusion study, an internal standard solution, which contained 30 blood samples were deproteinized by sulfosalicylic acid, and concentration: 12°C by means of a heating lamp. These vital signs were continuously monitored at mass-to-charge ratios of 234, 235, 239, and 240 for Phe enrichment and at mass-to-charge ratios of 200 and 207 for proline enrichment. L-[^13C]Phe enrichment was corrected for the contribution of the abundance of isotope-pomers of lower weight to the apparent enrichment of isotope-pomerons with larger weight, and a skew correction factor was applied (15). Isotope enrichment was expressed as the tracer-to-trace ratio for the internal standard method and as mole percent excess for calculation of protein kinetics and AA transport.

Plasma insulin concentration was determined by the microparticle enzyme immunoassay technique (1). AA concentrations (except proline) in plasma and in the free pool in skin wound were determined on an HPLC system (Waters 2690 HPLC system; Waters, Milford, MA) equipped with a Zorbax SB-C18 column. Blood glucose concentration was measured on a glucose/lactate analyzer (model 2300; Yellow Springs Instrument, Yellow Springs, OH). Blood hemoglobin (Hb) concentration was measured on an automated hematolysis analyzer (model JT3; Coulter, Hialeah, FL).

**Calculations.** Protein and Phe kinetics in the scalded skin were calculated from the following equations

\[
\text{inflow} = C_A \times BF
\]

**inward transport**

\[
= \frac{(E_{\text{sk}} - E_V)(E_A - E_{\text{sk}})}{C_A + C_V} \times BF
\]

**a-v shunting**

\[
= -\frac{(E_{\text{sk}} - E_V)(E_A - E_{\text{sk}})}{C_A + C_V} \times BF
\]

**outward transport**

\[
= \frac{(E_{\text{sk}} - E_V)(E_A - E_{\text{sk}})}{C_A + C_V} \times BF
\]

**outflow**

\[
= C_V \times BF
\]

**NB**

\[
= (C_A - C_V) \times BF
\]

**S_{\text{total}}**

\[
= \frac{(E_A \times C_A - E_V \times C_V)}{E_{\text{sk}} \times BF}
\]

**S_{\text{blood}}**

\[
= \frac{(E_A \times C_A - E_V \times C_V)}{E_{\text{sk}} \times BF}
\]

**synthesis from breakdown-derived AAs**

\[
= S_{\text{total}} - S_{\text{blood}}
\]

**breakdown releasing AAs into blood**

\[
= S_{\text{blood}} - NB
\]

where \(E_A\), \(E_V\), and \(E_{\text{sk}}\) are Phe enrichment in the arterial blood, venous blood, and free pool in skin wound; \(C_A\) and \(C_V\) are Phe concentration in the arterial and venous blood calculated by the internal standard method (20); \(BF\) is blood flow rate in the scalded ear; and \(NB\) is net balance. Equations 1–7 and 10 are published in our previous publications (27). Inflow is the rate of AA entering the ear via the artery; inward transport is the rate of delivery from the artery to the free pool in skin wound; a-v shunting is the rate of delivery directly from artery to vein; outward transport is the rate of delivery from the free pool in skin wound to ear vein; and outflow is the rate of AA exit via vein. Total synthesis is the rate of protein synthesis using AAs from blood and endogenous protein breakdown; total breakdown is the rate of AAs entering the free pool in skin wound from endogenous protein breakdown. Thus the total rate of appearance (total \(R_a\) in
the free pool in skin wound is the sum of inward transport and total breakdown. Equations 8 and 11 are derived from
Galin et al. (8). Because the arterial enrichment is used as precursor enrichment, Eq. 8 reflects the rate of synthesis from
blood-derived AAs (S_blood), which does not include synthesis from breakdown-derived AAs (S_breakdown); Eq. 11 reflects the rate of
AA release from breakdown into the venous blood (B_breakdown), which does not include reincorporation into protein. Consequently, S_breakdown is the difference between S_total and S_blood (i.e., Eq. 9), which is also referred to as intracellular cycling.

The capillary flow measured from the colored microsphere method was calculated by the following equation: flow rate = \[(\text{total tissue spheres})/(\text{tissue weight in g} \times \text{reference spheres}\cdot\text{ml}^{-1}\cdot\text{min}^{-1})\] (4).

Statistics. Data are expressed as means ± SD. Differences between two groups were evaluated by Student’s t-test. Differences among the four groups were evaluated using one-way ANOVA. Post hoc testing was accomplished using the nonpaired Student’s t-test combined with Bonferroni’s inequalities. A P value < 0.05 was considered statistically significant.

RESULTS

The rabbits ate little food during the first 48 h after injury. Thereafter, the food intake increased gradually. On the 5th and 6th days, they all consumed 150 g rabbit chow/day as before the injury. Table 1 lists the general characteristics of the animals. There were no significant differences in body weight, scalld time, rectal temperature, arterial blood O_2 saturation, or blood flow rate in scaldd skin among groups. In the AA group, the heart rate was significantly (P = 0.004) slower than in the insulin and insulin-AA groups. The mean arterial blood pressure was significantly (P = 0.02–0.04) lower in the insulin group than in the control and AA groups. The physiological conditions in individual rabbits remained stable during the tracer infusion, as demonstrated by the small percentages of coefficients of variation (CV) of the measured vital parameters in individual rabbits (n = 32): CV = 0.6 ± 0.4% for rectal temperature; CV = 5.7 ± 3.0% for heart rate; CV = 5.3 ± 2.6% for mean arterial blood pressure. The general characteristics of the four rabbits in the microsphere group were not included in the statistical analysis, but this group had characteristics similar to those of the other groups (Table 1).

Because of variable wound edema, the weight of scaldd skin did not entirely reflect skin mass. We dissected five pairs of normal and scaldd ears on the 7th day after injury. The wet weights of normal and scaldd ear skin were 13.1 ± 0.8 and 20.1 ± 3.5 g, respectively (P = 0.0025). After baking in an oven at 80°C for 72 h, the dry weights were not significantly different (4.14 ± 0.42 g for normal skin and 4.36 ± 0.66 g for scaldd skin; P = 0.15). Thus we used the contralateral normal ear to estimate skin weight in the scaldd ear to avoid the complication of edema. The ear weight was multiplied by 0.78 because we previously determined that the skin accounts for 78% of ear weight, with the remaining being ear cartilage (23).

Blood glucose concentrations were 9.9 ± 1.6 and 11.9 ± 0.9 mmol/l in the control and AA groups, respectively. These values represented a hyperglycemic response, because we reported in our previous publication (25) that the blood glucose concentration in normal conscious rabbits after an overnight fast was 3.8 ± 0.3 mmol/l. In the insulin and insulin-AA groups, euglycemia (5.3 ± 0.5 and 5.6 ± 0.8 mmol/l, P > 0.05) was maintained by glucose infusion at 7.9 ± 3.0 and 7.6 ± 1.9 mg·kg⁻¹·min⁻¹ (P > 0.05), respectively. Plasma insulin concentration was not significantly different between control and AA groups (26 ± 26 and 40 ± 21 pmol/l in control and AA groups, respectively, P > 0.05). Plasma insulin concentrations were raised to 1,613 ± 158 and 1,680 ± 368 pmol/l (P > 0.05) in the insulin and insulin-AA groups, respectively.

Plasma AA concentrations are presented in Table 2. Compared with the control group, infusion of insulin significantly (P < 0.05 or P < 0.01) decreased plasma essential amino acid (EAA) concentrations (except tryptophan and methionine). Although the concentrations of some nonessential amino acids (NEAA) were also decreased in the insulin group (i.e., aspartic acid/asparagine, serine, alanine, and proline), the total amount of NEAA was not significantly (P = 0.47) different from that in the control group. In contrast, the infusion of AAs significantly (P < 0.01) increased almost all of the plasma AA concentrations (except tyrosine and aspartic acid/asparagine). In the insulin-AA group, the total EAA concentration was significantly (P < 0.01) greater than that in the control group but less than in the AA group. AA concentrations in the free pool in skin wound were not significantly (P = 0.09 for EAA and P = 0.25 for NEAA) different among groups. The AA concentrations in skin wound

### Table 1. General characteristics of animals

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, kg</th>
<th>Scalld Time, s</th>
<th>Rectal Temperature, °C</th>
<th>Heart Rate, beats/min</th>
<th>MAP, mmHg</th>
<th>Arterial O_2 Saturation, %</th>
<th>Ear Blood Flow, ml·100 g⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>4.6 ± 0.3</td>
<td>3.3 ± 0.2</td>
<td>39.4 ± 0.4</td>
<td>201 ± 20</td>
<td>75 ± 7</td>
<td>98.0 ± 3.1</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>Insulin</td>
<td>8</td>
<td>4.5 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>39.2 ± 0.1</td>
<td>235 ± 37</td>
<td>64 ± 7*</td>
<td>99.8 ± 0.2</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>AA</td>
<td>7</td>
<td>4.2 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>39.2 ± 0.5</td>
<td>181 ± 17†</td>
<td>75 ± 6†</td>
<td>98.6 ± 3.2</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>Insulin-AA</td>
<td>7</td>
<td>4.5 ± 0.3</td>
<td>3.3 ± 0.1</td>
<td>39.3 ± 0.4</td>
<td>237 ± 31‡</td>
<td>72 ± 11</td>
<td>99.9 ± 0.1</td>
<td>35 ± 11</td>
</tr>
<tr>
<td>Microspheres</td>
<td>4</td>
<td>4.4 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>38.7 ± 0.8</td>
<td>182 ± 17</td>
<td>Not measured</td>
<td>See text</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD; n, no. of rabbits. Values of rectal temperature, mean arterial blood pressure (MAP), heart rate, and the rates of blood flow in the scaldd ear are averages during the 120–240 min of isotope infusion. Arterial O_2 saturation, arterial blood oxygen saturation; AA, amino acid. *P < 0.05 vs. control; †P < 0.05 vs. insulin; ‡P < 0.05 vs. AA. Because the microsphere group had only 4 rabbits and was used to determine the wound capillary flow, this group is not included in the statistical analysis with other groups.
were consistently higher \((P < 0.01)\) than those in plasma (Fig. 2, A and B).

Arterial Phe enrichment was at an isotopic plateau throughout the 150–240 min of the tracer infusion. Phe enrichment and concentration in the arterial and venous blood and Phe enrichment in the free pool in skin wound are presented in Table 3. The Phe concentrations in the ear-venous blood have been corrected for water loss from the wound surface. Because Hb remains in the vascular compartment when water evaporates, the a-v difference of Hb reflects the extent of water loss. We measured 25 pairs of arterial and ear-venous blood samples for Hb concentration in the present study. The Hb concentrations were 118.6 ± 2.6 and 123.9 ± 2.8 g/l \((P < 10^{−6}\) by paired t-test) in the arterial and venous blood, respectively, and the corresponding blood flow rate was 32.7 ± 0.8 ml·100 g^−1·min^−1. These data mean that, to account for the water loss from the wound surface, the measured venous Phe concentration should be multiplied by 95.08%. Because the water loss from the 7-day scalded skin can be considered constant under the same experimental conditions, the a-v difference in Hb concentration was inversely related to the blood flow rate. Therefore, we corrected the venous Phe concentration for the blood flow rate in each rabbit as follows: correction factor = \(1 - (32.7 × 4.92\%BF)\), where 32.7 is the average blood flow rate (ml·100 g^−1·min^−1) at the time of collection of the a-v blood for Hb measurement; the percent increase in Hb concentration from the artery to vein was 4.92%.

Protein kinetics in the skin wound calculated from Eqs. 6–11 are presented in Table 4. The insulin-AA group had the greatest value of net balance \((1.4 ± 5.2 \mu mol·100 g^−1·h^−1)\), which was significantly greater \((P = 0.02\) and \(P = 0.001)\) than those in the control and insulin groups \((-6.5 ± 4.5\) and \(-10.6 ± 6.6 \mu mol·100 g^−1·h^−1\), respectively). The difference in net balance between the control and insulin groups was not significant \((P = 0.27)\). The differences in total protein synthesis \((S_{total})\) did not reach statistical significance \((P = 0.07)\) between groups. The rate of total protein breakdown \((B_{total})\) in the insulin group was significantly \((P = 0.01–0.02)\) greater than in the control and insulin-AA groups. In the AA group, the rate of protein synthesis from blood-derived AAs \((S_{blood})\) was significantly \((P = 0.04)\) greater than in the control and insulin groups. The difference between the control and insulin-AA groups did not reach significance \((P = 0.09)\). The changes of the rates of AAs release into blood from protein breakdown \((B_{blood})\) did not reach significance between groups \((P = 0.07)\). The rate of intracellular cycling \((S_{breakdown})\) in the insulin group was significantly greater than that in other groups \((P = 0.004\) vs. control, \(P = 0.02\) vs. AA, and \(P = 0.03\) vs. insulin-AA).

Table 5 presents the values of Phe transport that were calculated from Eqs. 1–5. Both the Phe inflow into the ear via the artery and the rate of inward transport from blood to the free pool in skin wound were significantly \((P = 0.01–0.005)\) greater in the AA group than the corresponding rates in the control and insulin groups. There was a significant difference between the rates of outward transport in the AA and insulin-AA groups \((P = 0.049)\) but not between the control and AA groups \((P = 0.07)\). In the insulin group, the contribution of inward transport to total \(R_a\) in the free pool in skin wound (inward transport/total \(R_a\)) was significant.

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**Table 2. Plasma amino acid concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Control ((n = 10))</th>
<th>Insulin ((n = 8))</th>
<th>AA ((n = 7))</th>
<th>Insulin-AA ((n = 7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsX</td>
<td>54 ± 12</td>
<td>27 ± 4</td>
<td>70 ± 16</td>
<td>45 ± 23</td>
</tr>
<tr>
<td>GIX</td>
<td>591 ± 62</td>
<td>523 ± 155</td>
<td>970 ± 42†</td>
<td>867 ± 123‡</td>
</tr>
<tr>
<td>Ser</td>
<td>236 ± 42</td>
<td>109 ± 20</td>
<td>739 ± 299†</td>
<td>761 ± 226‡</td>
</tr>
<tr>
<td>His</td>
<td>110 ± 14</td>
<td>72 ± 10</td>
<td>236 ± 61†</td>
<td>209 ± 47‡</td>
</tr>
<tr>
<td>Gly</td>
<td>608 ± 130</td>
<td>628 ± 138</td>
<td>2,175 ± 440‡</td>
<td>2,269 ± 552‡</td>
</tr>
<tr>
<td>Arg</td>
<td>131 ± 24</td>
<td>52 ± 17</td>
<td>510 ± 72‡</td>
<td>417 ± 84‡</td>
</tr>
<tr>
<td>Ala</td>
<td>160 ± 27</td>
<td>88 ± 27</td>
<td>687 ± 175‡</td>
<td>587 ± 84‡</td>
</tr>
<tr>
<td>Tyr</td>
<td>38 ± 25</td>
<td>25 ± 12</td>
<td>42 ± 12‡</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>Pro</td>
<td>187 ± 28</td>
<td>68 ± 11</td>
<td>389 ± 90†</td>
<td>318 ± 63§</td>
</tr>
<tr>
<td>Thr</td>
<td>129 ± 28</td>
<td>42 ± 13</td>
<td>360 ± 108†</td>
<td>210 ± 20‡</td>
</tr>
<tr>
<td>Val</td>
<td>197 ± 39</td>
<td>69 ± 14</td>
<td>443 ± 41‡</td>
<td>215 ± 38‡</td>
</tr>
<tr>
<td>Met</td>
<td>19 ± 4</td>
<td>8 ± 4</td>
<td>112 ± 28‡</td>
<td>78 ± 9‡</td>
</tr>
<tr>
<td>Try</td>
<td>27 ± 8</td>
<td>29 ± 4</td>
<td>47 ± 19‡</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>Phe</td>
<td>69 ± 7</td>
<td>33 ± 2‡</td>
<td>100 ± 30†</td>
<td>81 ± 12†</td>
</tr>
<tr>
<td>Ile</td>
<td>77 ± 17</td>
<td>27 ± 6</td>
<td>214 ± 26‡</td>
<td>110 ± 22‡</td>
</tr>
<tr>
<td>Leu</td>
<td>154 ± 30</td>
<td>54 ± 10†</td>
<td>335 ± 48‡</td>
<td>159 ± 29‡</td>
</tr>
<tr>
<td>Lys</td>
<td>134 ± 28</td>
<td>72 ± 18</td>
<td>378 ± 43‡</td>
<td>237 ± 79‡</td>
</tr>
<tr>
<td>BCAA</td>
<td>429 ± 85</td>
<td>150 ± 29</td>
<td>992 ± 112‡</td>
<td>484 ± 88‡</td>
</tr>
<tr>
<td>EAA</td>
<td>806 ± 126</td>
<td>334 ± 59‡</td>
<td>1,988 ± 233‡</td>
<td>1,128 ± 144‡</td>
</tr>
<tr>
<td>NEAA</td>
<td>2,096 ± 248</td>
<td>1,591 ± 201</td>
<td>5,818 ± 1,245‡</td>
<td>5,506 ± 882‡</td>
</tr>
<tr>
<td>TAA</td>
<td>2,902 ± 330</td>
<td>1,925 ± 225</td>
<td>7,806 ± 1,376‡</td>
<td>6,634 ± 777‡</td>
</tr>
</tbody>
</table>

Data are means ± SD in \(\mu M\); \(n\), no. of rabbits. AsX, sum of asparagine and aspartic acid; GIX, sum of glutamine and glutamic acid; BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA, nonessential amino acids; TAA, total amino acids. \(*P < 0.05\) vs. control; †\(P < 0.05\) vs. insulin; ‡\(P < 0.05\) vs. AA.
cantly \((P = 0.001)\) less than in other groups. In contrast, the contribution of breakdown (breakdown/total \(R_a\)) in the insulin group was significantly \((P = 0.001)\) greater than in the other groups. In the AA group, the ratio inward transport/total \(R_a\) was significantly \((P = 0.003)\) greater than in the control group and the ratio breakdown/total \(R_a\) was significantly \((P = 0.03)\) smaller.

In the microsphere group, the measured capillary flow rates in the right and left kidneys were \(4.57 \pm 1.06\) and \(4.57 \pm 1.30\) ml·g\(^{-1}\)·min\(^{-1}\) \((P = 0.15)\), indicating that the injected microspheres were uniformly distributed in the blood stream. Six measurements were performed when the carotid artery was not clamped, and the blood flow rate measured by the ultrasonic blood flowmeter was \(99.1 \pm 25.9\) ml·100 g\(^{-1}\)·min\(^{-1}\). Another six measurements were performed during the arterial clamp, and the recorded blood flow rate from the flowmeter was \(32.2 \pm 5.6\) ml·100 g\(^{-1}\)·min\(^{-1}\). The corresponding rates of capillary flow measured from the microsphere technique were \(29.9 \pm 5.9\) and \(16.5 \pm 3.4\) ml·100 g\(^{-1}\)·min\(^{-1}\) without and with clamping, respectively. Because we placed a catheter in the left carotid artery, the blood flow rate in the normal skin of the left ear was reduced. Thus we used the capillary flow of normal ear skin of \(3.4 \pm 0.8\) ml·100 g\(^{-1}\)·min\(^{-1}\) from our previous study \(26)\) for comparison. The capillary flow rates in the scalded skin were 8.8- and 4.9-fold the normal skin rate without the arterial clamp and during the arterial clamp, respectively.

**DISCUSSION**

To repair skin defects, new proteins have to be deposited in the wounded area to restore skin integrity. Therefore, the most important parameter of wound protein kinetics is the net protein balance. In the present study, the infusion of either insulin or AAs alone failed to increase net protein balance in the skin wound. Only combined infusion of insulin and AAs significantly increased net protein balance, indicating an anabolic effect on protein in the skin wound. These data support an interactive effect rather than an additive effect of insulin and abundant AA supply on protein metabolism in the skin wound, because the combined effect was greater than the sum of their individual effects.

Whereas insulin alone was not sufficient to induce an anabolic effect on protein metabolism, it stimulated protein turnover in the skin wound (Table 4). To obtain an insight into insulin’s effect on wound protein synthesis, we can consider protein synthesis as being composed of two components (3). One component involves incorporation of AAs from the arterial blood, and the other component involves incorporation of AAs from endogenous protein breakdown within the tissue (i.e., intracellular cycling). Similarly, the breakdown of protein can also be divided into the following two components: one component releases AAs into venous blood, and the other component releases AAs for reincorporation into protein. An increase in reutilization of AAs from protein breakdown will never lead to an anabolic state, since 100% reutilization would yield a zero balance. Only an increase in the use of blood-derived AA for synthesis could lead to an improved net balance, provided that the component of breakdown that releases AAs in blood is not concomitantly increased to the same extent. In the insulin group, the component of synthesis using blood-derived AAs was not increased, but the component of synthesis using breakdown-derived AAs was significantly increased. Thus, although insulin infusion alone increased the synthetic capacity, it was not able to reduce net protein loss in the skin wound.

The protein kinetic data in the insulin group were supported by the Phe transport data and AA concentrations in the free pool in skin wound. Insulin infusion

**Table 3. Phenylalanine enrichment and concentration in blood and phenylalanine enrichment in the free amino acid pool in skin wound**

<table>
<thead>
<tr>
<th></th>
<th>(E_A)</th>
<th>(E_V)</th>
<th>(E_{SK})</th>
<th>(C_A)</th>
<th>(C_V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(0.0921 \pm 0.0051)</td>
<td>(0.0819 \pm 0.0043)</td>
<td>(0.0296 \pm 0.0051)</td>
<td>(66.5 \pm 5.2)</td>
<td>(69.5 \pm 6.6)</td>
</tr>
<tr>
<td>Insulin</td>
<td>(0.0891 \pm 0.0117)</td>
<td>(0.0719 \pm 0.0100)</td>
<td>(0.0179 \pm 0.0035)</td>
<td>(50.2 \pm 4.9)</td>
<td>(55.5 \pm 5.4)</td>
</tr>
<tr>
<td>AA</td>
<td>(0.0683 \pm 0.0063)</td>
<td>(0.0622 \pm 0.0051)</td>
<td>(0.0282 \pm 0.0071)</td>
<td>(116.9 \pm 13.5)</td>
<td>(120.1 \pm 14.9)</td>
</tr>
<tr>
<td>Insulin-AA</td>
<td>(0.0754 \pm 0.0082)</td>
<td>(0.0697 \pm 0.0055)</td>
<td>(0.0289 \pm 0.0025)</td>
<td>(95.6 \pm 8.3)</td>
<td>(95.2 \pm 9.1)</td>
</tr>
</tbody>
</table>

Data are means \(\pm SD\); \(n\), no. of rabbits; \(E_A\), \(E_V\), and \(E_{SK}\), phenylalanine enrichment (mole percent excess) in arterial blood, venous blood, and free AA pool in skin wound, respectively. \(C_A\) and \(C_V\), concentration of phenylalanine (\(\mu\)mol/l) in arterial and venous blood, respectively.

**Table 4. Protein kinetics in skin wound**

<table>
<thead>
<tr>
<th></th>
<th>Total Synthesis</th>
<th>Total Breakdown</th>
<th>Net Balance</th>
<th>(S_{blood})</th>
<th>(B_{blood})</th>
<th>Intracellular Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(32.7 \pm 13.5)</td>
<td>(39.2 \pm 12.1)</td>
<td>(-6.5 \pm 4.5)</td>
<td>(10.3 \pm 3.9)</td>
<td>(16.8 \pm 3.8)</td>
<td>(22.5 \pm 9.9)</td>
</tr>
<tr>
<td>Insulin</td>
<td>(50.6 \pm 16.2)</td>
<td>(61.2 \pm 16.0^*)</td>
<td>(-10.6 \pm 6.6)</td>
<td>(10.2 \pm 3.6)</td>
<td>(20.8 \pm 7.4)</td>
<td>(40.5 \pm 13.4^*)</td>
</tr>
<tr>
<td>AA</td>
<td>(40.4 \pm 10.2)</td>
<td>(47.1 \pm 13.5)</td>
<td>(-6.7 \pm 6.2)</td>
<td>(16.3 \pm 5.1^\dagger)</td>
<td>(22.9 \pm 8.8)</td>
<td>(24.1 \pm 6.7^\dagger)</td>
</tr>
<tr>
<td>Insulin-AA</td>
<td>(40.5 \pm 12.5)</td>
<td>(39.0 \pm 14.4^\ddagger)</td>
<td>(1.4 \pm 5.2^\ddagger)</td>
<td>(15.4 \pm 4.5)</td>
<td>(13.9 \pm 7.0)</td>
<td>(25.1 \pm 8.4^\dagger)</td>
</tr>
</tbody>
</table>

Data are means \(\pm SD\) in \(\mu\)mol Phe·100 g\(^{-1}\)·h\(^{-1}\); \(n\), no. of rabbits. Total synthesis is total rate of protein synthesis from blood-derived AAs \(S_{blood}\) and from breakdown-derived AAs. Total breakdown \(B_{total}\) is the rate of AAs entering the free pool in skin wound from endogenous protein breakdown. The breakdown-derived AAs are either released into blood \(B_{blood}\) or reincorporated into protein. Intracellular cycling is the rate of synthesis from breakdown-derived AAs. \(^*P < 0.05\) vs. control; \(^\dagger P < 0.05\) vs. insulin; \(^\ddagger P < 0.05\) vs. AA.
alone did not stimulate the inward transport of Phe from arterial blood into the free pool in skin wound (Table 5). Furthermore, in the free pool in skin wound, more Phe came from endogenous breakdown, and less Phe came from inward transport in the insulin group than in other groups. These findings are consistent with the increased intracellular cycling, which uses breakdown-derived AAs for protein synthesis. The concentrations of both EAA and NEAA in the free pool in skin wound were not lower in the insulin group than in other groups (Fig. 2, A and B), which also supports the absence of an increase in the net deposit of AAs into wound protein. On the basis of the above findings, we propose that the infusion of insulin stimulates the process of wound protein synthesis. However, in the absence of exogenous AAs, insulin infusion causes a systemic hypoaminoacidemia, so that, as a result, the inward transport of AAs from artery to the free pool in skin wound is limited. The increased demand for AAs for synthesis and limited availability of AAs from blood trigger the acceleration of protein breakdown to maintain the intracellular concentrations of AAs, which would otherwise be reduced by the stimulation of synthesis. The outcome is that the rate of protein turnover is accelerated and the net balance is not increased.

In the AA group, the plasma AA concentrations increased to over twofold the control value (Table 2), which is consistent with increases in both inflow via the artery and inward transport from the artery to the free pool in skin wound (Table 5). When the contributions of Phe to protein synthesis from the two sources are compared, AA infusion increased the contribution of Phe from inward transport and decreased the contribution of Phe from endogenous protein breakdown. However, the net balance did not increase. The most likely explanation is that the component of protein breakdown that releases AAs into blood was concomitantly increased. In fact, the rate of Bblood was greatest in the AA group, although the difference did not reach significance ($P = 0.07$, see Table 4). The rate of outward transport was also greatest in the AA group (Table 5). Thus it seems that the changes in synthesis and breakdown in the AA group were basically parallel, which may explain why there was no improvement of net balance.

The combined infusion of insulin and abundant AA supply increased net protein balance in skin wound. In general, an anabolic effect could be induced by an increase in synthesis, decrease in breakdown, or both. Because neither the increase in total synthesis nor the decrease in total breakdown reached statistical significance in the insulin-AA group, we cannot be certain of the mechanism responsible for the increase in net balance. Most likely, there were additive effects of changes in synthesis and breakdown that accounted for the improved net balance.

When comparison is made between the insulin-AA and insulin groups, the insulin-AA group had significantly lower rates of total protein breakdown and intracellular cycling. On the other hand, when comparison is made between the insulin-AA and AA groups, the insulin-AA group had a significantly lower rate of outward transport. These differences suggest that the combined infusion of insulin and abundant AAs might enable more efficient use of blood-derived AAs for synthesis while simultaneously inhibiting the loss of AAs into blood.

Although the exact mechanism is not straightforward, the net anabolic effect of insulin and exogenous AA supply on protein metabolism in skin wound is conclusive. This is consistent with the notion that exogenous AA supply plays an important role in insulin’s anabolic effect on protein metabolism in muscle (17, 19, 21, 22). In the present experiment, we also determined the protein kinetics in the skeletal muscle by using the hindlimb as an a-v unit. The responses of muscle protein were basically the same as those of wound protein: only combined infusion of insulin and AAs significantly increased the net protein balance in muscle (data not shown).

In the present study, we reduced the rate of wound blood flow to within four- to fivefold the normal skin rate by clamping the carotid artery. This procedure increased the a-v difference of Phe enrichment to 10–20% of the arterial value (see Table 3), which enabled an accurate determination of the a-v difference of Phe enrichment under the hyperaminoacidemic condition. Even when the flow was clamped, however, a-v shunting still accounted for 83–88% of Phe inflow (estimated from Table 5). Thus at least some of the arterial inflow likely did not provide nutritive flow to the tissues. We used the microsphere technique to better assess the rate of capillary flow in the skin wound. The results showed that, during the arterial clamping, the capillary flow in the skin wound was approximately fivefold the normal skin rate, so that there was sufficient blood perfusion in the skin wound to enable the delivery of nutrients. This notion was confirmed by the rate of

### Table 5. Phenylalanine transport in skin wound

<table>
<thead>
<tr>
<th>Phenylalanine, μmol·100 g⁻¹·h⁻¹</th>
<th>n</th>
<th>Inflow via artery</th>
<th>Inward transport</th>
<th>a-v Shunting</th>
<th>Outward transport</th>
<th>Total $R_a$</th>
<th>Inward TransPort/Total $R_a$</th>
<th>Breakdown/Total $R_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>145 ± 21</td>
<td>18 ± 5</td>
<td>127 ± 18</td>
<td>25 ± 5</td>
<td>57 ± 16</td>
<td>0.32 ± 0.05</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>Insulin</td>
<td>8</td>
<td>95 ± 19</td>
<td>16 ± 6</td>
<td>79 ± 16</td>
<td>26 ± 10</td>
<td>77 ± 20</td>
<td>0.21 ± 0.05*</td>
<td>0.79 ± 0.05*</td>
</tr>
<tr>
<td>AA</td>
<td>7</td>
<td>222 ± 59†</td>
<td>33 ± 15†</td>
<td>190 ± 59†</td>
<td>39 ± 18</td>
<td>80 ± 26</td>
<td>0.40 ± 0.07†</td>
<td>0.60 ± 0.07†</td>
</tr>
<tr>
<td>Insulin-AA</td>
<td>7</td>
<td>200 ± 57†</td>
<td>24 ± 8</td>
<td>176 ± 53†</td>
<td>22 ± 11†</td>
<td>63 ± 22</td>
<td>0.39 ± 0.05†</td>
<td>0.61 ± 0.05†</td>
</tr>
</tbody>
</table>

Data are means ± SD; n, no. of rabbits. Total $R_a$, total rate appearance of AA in the free pool in skin wound, which is the sum of inward transport and total breakdown. *$P < 0.05$ vs. control; †$P < 0.05$ vs. insulin.
protein synthesis. In the control group, the rate of wound protein synthesis (32.7 ± 4.3 μmol·100 g⁻¹·h⁻¹) was 23% greater (P > 0.05) than the control value in our previous experiment (26.6 ± 2.8 μmol·100 g⁻¹·h⁻¹) in which the wound blood flow rate was not restricted (24). It is interesting that the rate of the net protein loss in skin wound of the controls was significantly (P < 0.05) lower than the previous value (−23.1 ± 21.4 μmol·100 g⁻¹·h⁻¹) when blood flow rate was not restricted (24). In the previous experiment, we also found that the improvement of protein net balance in skin wound during infusion of insulin and low-dose AAs was accompanied by a 41–44% decrease of wound blood flow rate (24). It may be that a rapid rate of blood flow increases the outward transport of AAs resulting from a more extensive equilibration between the high intracellular concentrations and the lower extracellular concentrations (see Fig. 2, A and B). Thus, although our experimental design did not enable us to assess the effect of blood flow on wound protein metabolism, the experimental conditions provided adequate nutritive flow. Furthermore, the blood flow was similar in all groups, so comparison of the metabolic effects of insulin and AAs was reasonable.

In summary, insulin’s anabolic effect on protein metabolism in skin wound required sufficient supply of exogenous AAs. Without exogenous AA supply, insulin stimulated only the rate of intracellular cycling of AAs. On the other hand, without insulin administration, infusion of a large dose of a balanced AA solution (10% Travasol) was insufficient to induce an anabolic response in the skin wound. Only the combined infusion of insulin and abundant AAs significantly increased net protein balance. These findings indicate an interactive rather than an additive effect of insulin and exogenous AA supply.

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