

Insulin's Effect on Synthesis Rates of Liver Proteins

A Swine Model Comparing Various Precursors of Protein Synthesis

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Insulin's effect on the synthesis of liver proteins remains to be fully defined. Previous studies using various surrogate measures of amino acyl-tRNA have reported variable results of insulin's effect on liver protein synthesis. We determined the effect of insulin with or without amino acid supplementation on the synthesis rates of liver proteins (tissue, albumin, and fibrinogen) using L-[1-¹³C]Leu as a tracer in 24 male miniature swine. In addition, we compared the isotopic enrichment of different precursors of liver proteins with that of amino acyl-tRNA using L-[1-¹³C]Leu and L-[¹⁵N]Phe as tracers. Although liver tissue fluid enrichment of [¹³C]Leu and [¹⁵N]Phe and that of plasma [¹³C]ketoisocaproic acid (KIC) were very similar to that of tRNA, plasma isotopic enrichment of both Leu and Phe were substantially higher ($P < 0.01$) and VLDL apolipoprotein-B₁₀₀ enrichment was lower ($P < 0.01$) than the respective amino acyl-tRNA enrichment. Plasma KIC enrichment most accurately predicted leucyl-tRNA enrichment, whereas plasma Leu enrichment was best correlated with that of tRNA. Neither insulin alone nor insulin plus amino acid infusion had an effect on liver tissue protein synthesis. In contrast, insulin alone decreased the albumin synthesis rate, and insulin with amino acids maintained the albumin synthesis rate. Insulin with or without amino acids inhibited the fibrinogen synthesis rate. These results, based on synthetic rates using amino acyl-tRNA, were consistent with those obtained using KIC or tissue fluid Leu or Phe as precursor pools. These studies demonstrated that plasma KIC enrichment is a convenient and reliable surrogate measure of leucyl-tRNA in liver. We also concluded that insulin has differential effects on the synthesis rates of liver proteins. Whereas insulin with or without amino acid supplement has no acute effect on the synthesis of liver tissue protein, insulin has a substantial inhibitory effect on fibrinogen synthesis. In contrast, insulin administration along with amino supplement is necessary to maintain albumin synthesis rate. *Diabetes* 50:947-954, 2001

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ApoB, apolipoprotein-B; FSR, fractional synthesis rate; KIC, ketoisocaproic acid; TCA, trichloroacetic acid.

The application of accurate and sensitive isotopic tracer techniques for measuring the fractional synthesis rate (FSR) of protein is crucial when investigating the regulation of protein metabolism under various pathophysiological conditions (1-15). The FSR of tissue protein is derived by dividing the increment or slope of isotopic enrichment in proteins over time by its precursor enrichment at plateau; it is thus crucial to select the most accurate and reliable precursor for the calculation of FSR. Although the obligatory precursor of protein synthesis is amino acyl-tRNA, its measurement is not practical in most situations. Isotopic enrichment of tracers in tissue fluid has been shown to be highly correlated with that of tRNA (16) and has been applied to measuring the synthesis rates of muscle proteins (17), muscle mitochondrial proteins (18), and skin collagen (9). However, for measurement of liver protein synthesis, the sampling of human liver tissue is impractical in most cases, and thus the use of both tRNA and tissue fluid are not routinely feasible. So far, human studies measuring liver protein synthesis have used surrogate measures of liver tRNA, such as enrichment of [¹³C]Leu, [¹³C]ketoisocaproic acid ([¹³C]KIC), [¹⁵N]Phe in plasma, and [¹³C]Leu or [¹⁵N]Phe enrichments in VLDL apolipoprotein-B₁₀₀ (ApoB₁₀₀) (5,19,20). Of these, isotopic enrichment in VLDL ApoB₁₀₀ (a circulating protein mainly produced in the liver that has a relatively short half-life) has been proposed as theoretically the most accurate surrogate measure of liver amino acyl-tRNA (5,19,20). The rationale for this approach is that a protein that turns over rapidly reaches a plateau within a few hours, when its isotopic enrichment reaches the same level as that of its immediate precursor, which is amino acyl-tRNA. However, this hypothesis has not been tested in any previous studies. It is possible that part of the circulating VLDL ApoB₁₀₀ secreted into circulation during the isotope infusion is synthesized and stored before the isotope infusion is started. In such a situation, ApoB₁₀₀ enrichment is theoretically lower than that of tRNA. The current study was designed to determine the relationship of various precursor pools to amino acyl-tRNA under different physiological conditions and to infer which one best represents amino acyl-tRNA. We performed these

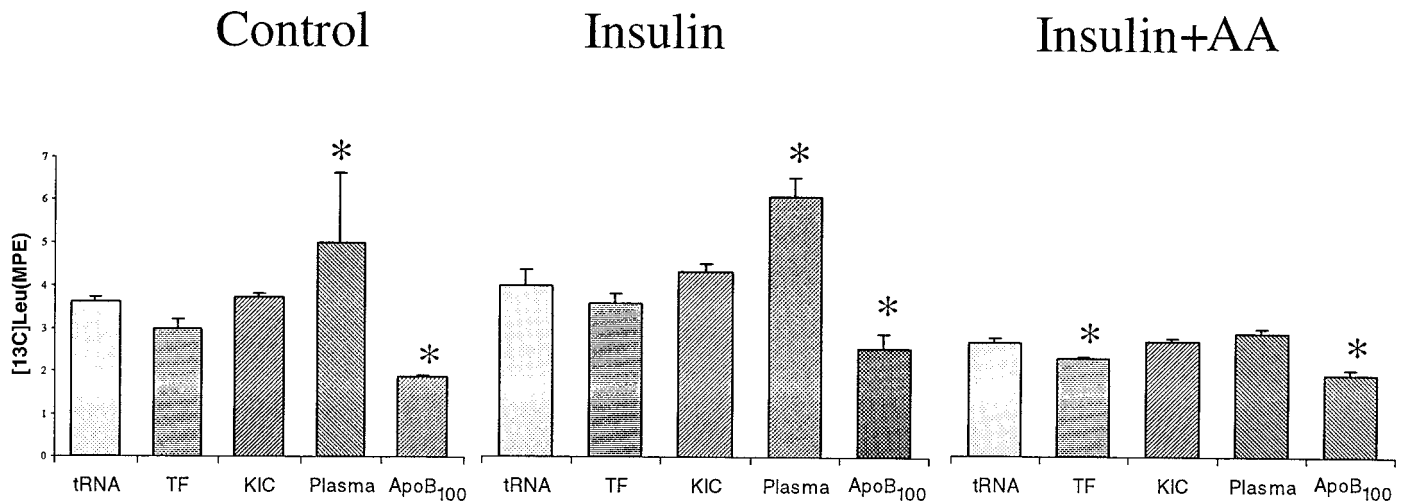


FIG. 1. Mean isotopic enrichment (molar percent excess [MPE]) of [^{13}C]leucyl-tRNA (tRNA), [^{13}C]Leu in tissue fluid (TF), plasma [^{13}C]KIC, plasma [^{13}C]Leu (plasma), and [^{13}C]Leu in VLDL ApoB₁₀₀ (ApoB₁₀₀). * $P < 0.05$ vs. tRNA.

studies in a swine model with various insulin and amino acid levels. This study also provided us with a unique opportunity to determine insulin's effect with or without amino acids on the synthesis rate of liver proteins measured using amino acyl-tRNA as the precursor pool.

Previous studies on insulin's effect on liver protein synthesis have reported conflicting results (12,21–25). In human studies, insulin infusion alone has been shown to decrease splanchnic protein synthesis in nondiabetic (26) and type 1 diabetic (27) individuals. However, insulin replacement in type 1 diabetic patients has been shown to increase gut mucosal protein synthesis (28), suggesting that most of the changes related to insulin in the splanchnic bed take place in the liver. It is also clear that although insulin replacement may stimulate synthesis of some liver proteins, such as albumin (23), synthesis of other liver proteins, such as fibrinogen, are inhibited (6). The precise role of amino acids versus insulin on liver protein synthesis also remains uncertain (12,22–25,29). To clearly define the role of insulin in regulating the synthesis rate of liver proteins, we used amino acyl-tRNA to measure the synthesis rates of both liver tissue proteins and liver secretory proteins such as albumin and fibrinogen. We specifically determined the effect of insulin alone and insulin plus amino acids on the synthesis rates of these liver proteins.

RESEARCH DESIGN AND METHODS

Animals. The 24 sexually mature male Hanford miniature swine (Charles River Laboratories, Wilmington, MA), weighing 30.0–42.7 kg (35.0 ± 2.9 kg), were studied after two days' acclimation after arrival. The animals were kept under controlled conditions (12:12 h light:dark cycle) and fed standard diet (Lean Grow 93; O'Lakes, Fort Dodge, IA). The study was approved by the Institutional Animal Care and Use Committee, and care and handling of the animals were in accordance with the National Institutes of Health guidelines for the use of experimental animals.

Experimental procedures. All studies were performed after an overnight fast with free access to tap water. The animals were given an intramuscular injection of ketamine (12.5 mg/kg body wt; Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (2 mg/kg body wt; Miles, Shawnee Mission, KS) as a tranquilizer and anesthesia. This was followed by continuous administration of isoflurane (1.25–2.25% isoflurane, USP; Abbott Laboratories, North Chicago, IL) delivered in oxygen at a rate of 5 l/min by a mouth mask to maintain a surgical level of anesthesia. The pigs were mechanically ventilated using a Harvard Respirator (Apparatus Dual Phase Control, model 613; Harvard Apparatus, South Natick, MA) throughout the entire study period. Ventilation was adjusted to a respiratory rate of 12–15 breaths per minute to

achieve an arterial carbon dioxide tension of 30–45 mmHg. A continuous electrocardiographic monitoring of the heart rate and rhythm was performed. The body temperature was maintained at 38–39°C by circulating water in a heating blanket. The anesthetic agents were chosen based on their lack of any known liver toxicities. The animals were then intubated through a tracheostomy with polyethylene catheters in the femoral vein and femoral artery. In four pigs, because of technical problems, the carotid artery was used as an alternative to the femoral artery for blood sampling. The abdominal cavity of each animal was opened through a short right subcostal incision. This incision was closed using clamps until sampling was started and was covered with drapes to avoid evaporation and heat loss.

All 24 pigs were randomly assigned to one of the three infusion protocols: 1) the saline group (control group; $n = 8$), given a normal saline infusion ($5\text{--}10 \text{ ml} \cdot \text{kg}^{-1} \text{ body wt} \cdot \text{h}^{-1}$); 2) the insulin infusion group (insulin group; $n = 8$), given an infusion of insulin ($0.7 \text{ mU} \cdot \text{kg}^{-1} \text{ body wt} \cdot \text{min}^{-1}$; Regular Iletin II purified pork insulin, Eli Lilly, Indianapolis, IN) and maintained in a euglycemic condition with a glucose clamp (20% dextrose; Baxter Healthcare, Deerfield, IL); and 3) the insulin plus amino acids group (insulin + AA group; $n = 8$), given an insulin infusion ($0.7 \text{ mU} \cdot \text{kg}^{-1} \text{ body wt} \cdot \text{min}^{-1}$), maintained in a euglycemic glucose condition, and given an amino acid infusion ($2 \text{ ml} \cdot \text{kg}^{-1} \text{ body wt} \cdot \text{h}^{-1}$; 10% Travasol; Baxter Healthcare).

Stable isotope infusions. All animals received an 8-h primed (bolus at time zero equivalent to the dose for 1 h) continuous infusion of L-[^{13}C]Leu and L-[^{15}N]Phe (Cambridge Isotope Laboratories, Woburn, MA) administered via the femoral vein (1.00 and $0.75 \text{ mg} \cdot \text{kg}^{-1} \text{ body wt} \cdot \text{h}^{-1}$, respectively).

Sample collection. Blood samples were collected from the artery at baseline and every hour from 4 to 8 h. These samples were used for analysis of the isotope enrichment in plasma. Plasma samples for analysis of glucagon, growth hormone, and insulin were collected at 4, 6, 7, and 8 h.

The blood glucose level was measured every 15 min during the entire study period using a One Touch II Blood Glucose Meter (Lifescan, Milpitas, CA), and plasma glucose levels were maintained at steady state by dextrose infusion. Blood samples were also taken every hour, and blood glucose was analyzed with a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA).

Through the abdominal incision, a wedge-formed biopsy specimen weighing ~ 1 g was taken from the right and anterior edge of the liver at 4, 6, and 8 h (Fig. 1). The biopsy sites in the liver were sutured to minimize bleeding. The liver samples were frozen immediately in isopentane chilled in liquid nitrogen. All samples were stored at -80°C until analyzed. Animals were killed at the completion of the study by an intravenous lethal dose of sodium pentobarbital (Sleepaway, Fort Dodge Laboratories).

Analytical procedures. The free amino acid concentrations in plasma were measured by an automated high-performance liquid chromatography system using precolumn O-phthalaldehyde derivatization (30).

Separation of VLDL ApoB₁₀₀. Plasma VLDL was separated through a density ultracentrifugation of 3 ml plasma (1.006 g/ml ; Beckman L-70 Ultracentrifuge; Beckman Instruments, Palo Alto, CA) at a speed of 40,000 rpm for 18 h at 10°C . Then ~ 2.5 ml VLDL from the upper layer of the Quick-Seal tube (Beckman Instruments) were aspirated into an 8-ml glass tube with screw cap. An equal amount of isopropanol was then added to precipitate ApoB₁₀₀ at 4°C overnight. The VLDL precipitant was centrifuged at 5,000 rpm for 30 min at 4°C the next morning. The supernatant was carefully poured out, and the

VLDL precipitant was washed with 2 ml 100% isopropanol and respun for 15 min. The supernatant was discarded, and the trace isopropanol residue within the VLDL precipitant was dried under nitrogen. Approximately 50 μ l 0.1N phosphate buffered saline was added to dissolve the precipitant. The VLDL precipitant was stored at -20°C until the VLDL ApoB₁₀₀ was separated by SDS-PAGE. SDS-PAGE slab gels (10%) were used for separating VLDL ApoB₁₀₀ under denaturing conditions using Bio-Rad II xi electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The run was performed at a 35-mA constant current at 15°C for 6–7 h. The protein bands were visualized by silver stain (Bio-Rad). The stained ApoB₁₀₀ bands were cut with a laboratory spatula, transferred into a glass tube, and washed overnight with constant shaking and then hydrolyzed in 1 ml 6N HCl for 18–20 h at 110°C . The hydrolysates were centrifuged at 3,000 rpm at 4°C for 15 min to pellet the acrylamide gel residues. The supernatant was transferred into another tube and dried under an N₂ stream and then diluted with 0.01N HCl. The amino acid hydrolysates were passed over a cation ion-exchange column containing \sim 1 ml AG 50W cation exchange resin (Bio-Rad). The retained amino acids were subsequently eluted with 3.5 ml 3 mol/l ammonium hydroxide and dried down (Speed-Vac Plus; Savant Instruments, Farmingdale, NY). The amino acid hydrolysates were stored at -80°C in 0.5 ml 0.01 M HCl before derivatization for mass-spectrometric analysis of the isotopic enrichments.

Amino acyl-tRNA isolation of liver tissue. Amino acyl-tRNA was isolated from liver tissue as previously described (16,31,32) with necessary modifications for the smaller sample size. Briefly, frozen liver tissue (\sim 250 mg) was powdered with the aid of a mortar and pestle in liquid nitrogen. The powder was further homogenized in a 1% SDS/50 mmol/l cacodylic acid buffer (pH 5.5, 10% wt/vol). The solution was extracted twice with phenol at room temperature; the RNA was precipitated from the aqueous supernatant by the addition of 0.1 vol of 3 mol/l sodium acetate and 2.5 vol of ethanol and stored overnight at -20°C . The pellet was washed twice with 70% ethanol, and the RNA fractions were dissolved in 0.1 mol/l sodium acetate–0.01 mol/l magnesium acetate buffer (pH 4.0). The insoluble residue was then removed by centrifugation at 110,000g for 1 h, and the RNA was precipitated from the resulting supernatant by ethanol. Amino acyl-tRNA was de-acylated by the addition of 50 mmol/l sodium bicarbonate (pH 9.0) and incubation at 37°C for 1 h. The solution was acidified (pH 2.0) by the addition of HCl, placed on ice for 15 min, and then centrifuged at 3,000g for 15 min. The supernatant containing the amino acids was stored at -80°C until mass-spectrometric analysis of isotopic enrichments.

Tissue fluid amino acids (acid-soluble fraction of liver tissue) (32). Liver tissue (\sim 50 mg) was homogenized in 1 mol/l perchloric acid. Samples were then centrifuged at 3,000g for 30 min. The supernatant containing the free pool of amino acids was removed and purified by means of a cation exchange column (AG 50W-X8 resin, 100–200 mesh, hydrogen form; Bio-Rad). The NH₄OH fraction was evaporated to dryness in a Speed Vac (Savant) for subsequent analysis by mass spectrometry.

Purification of fibrinogen. Fibrinogen was purified from plasma after VLDL separation. The technique used was as previously described (33). The fibrinogen clot was washed twice with water then digested with 6N HCl for 24 h at 110°C . The hydrolysates were further clarified with a cation ion exchange resin as for tissue fluid amino acids, stored at -80°C and analyzed for isotopic enrichment in a gas chromatograph/isotope ratio/mass spectrometer (34).

Purification of albumin (33). After centrifugation, 10% trichloroacetic acid (TCA) was added to 1 ml plasma, from which VLDL had been removed. The precipitate was then washed with 2 ml 2.5% TCA and centrifuged again. The supernatant was poured off. Then 2 ml alcohol were added and mixed by vortexing, and the solution was stored overnight. From this extraction, 1 ml of the alcohol solution was taken out and dried under vacuum. Then 1 ml 6N HCl was added to digest the precipitant. The hydrolysates were further cleaned with a cation ion exchange column and stored at -80°C (34).

Analysis of isotope enrichment. Amino acids isolated from amino acyl-tRNA, tissue fluid, and plasma samples were derivatized as their *N*-trifluoroacetyl isopropyl esters (32) for analysis of isotope enrichment by gas chromatography–mass spectrometry (Hewlett-Packard, Avondale, PA), with selected ion monitoring under negative ion chemical ionization conditions using ammonia as the reactant gas (32). Hydrolysates of albumin and fibrinogen were derivatized by first reacting them with 4 mol/l methanol-HCl and then were derivatized to their heptafluorobutyric ester for measurement of [¹³C]Leu enrichment by gas chromatography–isotope ratio mass spectrometry (Finigan-MAT, Bremen, Germany), as previously described (34).

Calculations. The FSRs of proteins were calculated using the following formula: FSR (%/h) = $[\text{I}_{\text{et}}/(\text{I}_{\text{ep}} \times t)] \times 100$, where I_{et} is the increment in isotopic enrichment in protein or slope (in the case of albumin and fibrinogen, of isotopic enrichment against time), I_{ep} is the precursor pool enrichment representing the average of multiple samples among the protein sampling periods, and *t* is the time between sampling points in hours.

TABLE 1
Plasma concentrations of glucose and insulin

| | Control | Insulin | Insulin + AA |
|------------------------|------------------|-------------------|-------------------|
| Glucose (mg/100 ml) | | | |
| Baseline | 92.0 \pm 4.2 | 97.3 \pm 5.0 | 107.4 \pm 6.5 |
| 4 h | 109.4 \pm 13.3 | 82.5 \pm 5.1 | 82.6 \pm 4.0 |
| 5 h | 95.2 \pm 7.8 | 87.3 \pm 5.5 | 95.6 \pm 4.7 |
| 6 h | 90.7 \pm 4.8 | 95.6 \pm 4.8 | 96.1 \pm 6.6 |
| 7 h | 80.6 \pm 3.6 | 91.6 \pm 3.3 | 92.5 \pm 6.4 |
| 8 h | 80.3 \pm 3.1 | 93.0 \pm 3.5 | 93.8 \pm 6.6 |
| Mean | 94.7 \pm 3.5 | 89.0 \pm 1.8 | 96.2 \pm 2.8 |
| Insulin (μ mol/l) | | | |
| 4 h | 1.69 \pm 0.37 | 29.39 \pm 2.39 | 33.20 \pm 2.15 |
| 6 h | 1.60 \pm 0.28 | 34.23 \pm 1.72 | 38.10 \pm 2.26 |
| 7 h | 1.85 \pm 0.49 | 38.00 \pm 2.96 | 41.95 \pm 2.40 |
| 8 h | 1.99 \pm 0.38 | 42.35 \pm 4.08 | 46.30 \pm 5.83 |
| Mean | 1.81 \pm 0.22 | 38.19 \pm 1.83* | 42.12 \pm 2.25* |

Data are means \pm SE. * $P < 0.0001$ (higher than control group).

Statistical methods. All data are presented as means \pm SE. The group differences for protein synthesis were assessed by analysis of variance and Fisher's protected least significant differences test.

The agreement of each surrogate precursor with amino acyl-tRNA was assessed by computing the absolute deviation from the tRNA value. Accuracy was compared between methods by comparing the absolute deviations using paired *t* tests.

The correlation between each surrogate precursor and tRNA was assessed using Pearson's correlation coefficient. A multivariate analysis to assess the predictive value of multiple precursors simultaneously in predicting tRNA was carried out using stepwise multiple regression analysis, stepping up, with $P < 0.05$ as the criterion for entry into the model.

RESULTS

Plasma glucose. Plasma glucose concentrations in all three groups were maintained within the normal range (80–120 mg/dl) and were not significantly different among the three groups during the study period (Table 1). However, the plasma insulin levels were higher in the insulin and insulin + AA groups than in the control group ($P < 0.0001$) (Table 1), whereas no significant difference was found between the two insulin groups.

Plasma amino acid concentrations. Most of the plasma amino acid concentrations were higher in the insulin + AA group than in the control and insulin groups (Table 2). Some of the amino acid levels were lower in the insulin group than in the control group.

Precursor pool enrichment. Figures 1 and 2 give the precursor pool enrichment values of the three groups of pigs. Plasma [¹³C]Leu enrichment was higher than that of leucyl-tRNA enrichment in the control and insulin groups, but not in the insulin + AA group. In contrast, [¹³C]Leu in VLDL ApoB₁₀₀ was lower than that of tRNA in all cases. [¹³C]Leu in tissue fluid was different from tRNA only in the insulin + AA group. [¹³C]KIC enrichment was not different from that of tRNA in any group.

In contrast, all other comparison deviations of tissue fluid and KIC showed significant differences among the groups (Table 3). The accuracy of each of the surrogate precursors was assessed by comparison of absolute deviation from the tRNA value and the ratios of the other precursors and tRNA value. The deviations of plasma amino acids (both [¹³C]Leu and [¹⁵N]Phe) and ApoB₁₀₀ were not significantly different. Both plasma amino acid enrichment and ApoB₁₀₀ enrichment values had the highest deviation from that of tRNA, although the deviation

TABLE 2
Plasma amino acid concentrations

| Amino acids | Control | Insulin | Insulin + AA | Significance |
|-------------|-----------|-------------|--------------|--------------|
| Thr | 175 ± 18 | 117 ± 9.0 | 510 ± 32 | *†‡ |
| Ser | 159 ± 19 | 151 ± 14 | 396 ± 34 | *† |
| Glu | 274 ± 25 | 387 ± 72 | 362 ± 39 | |
| Gln | 623 ± 131 | 649 ± 97 | 721 ± 41 | |
| Gly | 814 ± 97 | 1,137 ± 151 | 2,161 ± 169 | *†‡ |
| Ala | 492 ± 80 | 289 ± 27 | 1,111 ± 125 | *†‡ |
| Val | 410 ± 58 | 301 ± 30 | 873 ± 45 | *†‡ |
| Met | 48 ± 3.9 | 44 ± 5.5 | 220 ± 15 | *† |
| Ile | 178 ± 21 | 120 ± 14 | 576 ± 36 | *†‡ |
| Leu | 326 ± 32 | 255 ± 28 | 645 ± 40 | *†‡ |
| Tyr | 80 ± 11 | 60 ± 7.0 | 55 ± 5.0 | |
| Phe | 143 ± 22 | 126 ± 13 | 286 ± 27 | *† |
| Lys | 168 ± 23 | 118 ± 10 | 367 ± 22 | *†‡ |
| His | 88 ± 7.0 | 69 ± 10 | 152 ± 28 | |
| Arg | 204 ± 17 | 206 ± 21 | 571 ± 39 | *† |

Data are means ± SE in μmol/l average values of 4, 5, 6, 7, and 8 h taken in each animal. *Insulin + AA group higher than control group, $P < 0.001$; †insulin + AA group higher than insulin group, $P < 0.001$; ‡significant differences between control group and insulin group, $P < 0.05$.

was in the opposite direction. The lowest deviation from tRNA was for [¹³C]KIC enrichment, which was significantly different from that of [¹³C]Leu in tissue fluid ($P < 0.03$). When [¹⁵N]Phe was used as a tracer, the least deviation from tRNA was for [¹⁵N]Phe in tissue fluid.

In summary, plasma [¹³C]KIC is the most accurate predictor of [¹³C]Leu-tRNA enrichment, followed by tissue fluid [¹³C]Leu when L-[1-¹³C]Leu is used as a tracer. When [¹⁵N]Phe is used, a tracer tissue fluid [¹⁵N]Phe most accurately predicts the enrichment of tRNA. The accuracy was lowest in predicting tRNA enrichment using either plasma amino acid enrichment or VLDL ApoB₁₀₀ enrichment.

In regression analysis, the best correlation to tRNA was with plasma [¹³C]Leu enrichment ($r^2 = 0.830$) and plasma [¹⁵N]Phe ($r^2 = 0.703$). These were better than the correlation of tRNA to [¹³C]KIC ($r^2 = 0.698$), tissue fluid [¹³C]Leu ($r^2 = 0.585$), and ApoB₁₀₀ [¹³C]Leu ($r^2 = 0.482$) and ApoB₁₀₀ [¹⁵N]Phe ($r^2 = 0.383$) enrichment. However, the linear prediction equation has an intercept and slope of

TABLE 3
Median absolute deviation from tRNA and median ratios of other precursor pools to tRNA

| | [¹⁵ N]Phe | [¹³ C]Leu |
|--------------------------------------|-----------------------|-----------------------|
| Median absolute derivation | | |
| Value | | |
| Tissue fluid | 0.5128 | 0.4607 |
| Plasma | 1.6062 | 1.1625 |
| ApoB ₁₀₀ | 1.1622 | 1.2845 |
| KIC | — | 0.2516 |
| <i>P</i> value | | |
| Tissue fluid vs. plasma | <0.001 | 0.006 |
| Tissue fluid vs. ApoB ₁₀₀ | <0.001 | <0.001 |
| Plasma vs. ApoB ₁₀₀ | 0.607 | 0.281 |
| KIC vs. tissue fluid | — | 0.030 |
| KIC vs. plasma | — | <0.001 |
| KIC vs. ApoB ₁₀₀ | — | <0.001 |
| Median ratios | | |
| Value | | |
| Tissue fluid | 0.8947 | 0.8480 |
| Plasma | 1.4111 | 1.3311 |
| ApoB ₁₀₀ | 0.5753 | 0.6113 |
| KIC | — | 1.0696 |
| <i>P</i> value | | |
| Tissue fluid vs. placebo | 0.0001 | 0.0001 |
| Tissue fluid vs. ApoB ₁₀₀ | 0.0001 | 0.0001 |
| Plasma vs. ApoB ₁₀₀ | 0.0001 | 0.0001 |
| KIC vs. tissue fluid | — | 0.0001 |
| KIC vs. plasma | — | 0.0001 |
| KIC vs. ApoB ₁₀₀ | — | 0.0001 |

0.127 and 0.9194 for KIC and 1.015 and 0.5217 for plasma Leu, respectively. This suggests that the agreement with tRNA is closer with KIC than plasma Leu, consistent with the results based on accuracy estimation. In a stepwise multiple regression analysis for each study situation, none of the other variables came into the model once plasma amino acid enrichment (which has the best correlation to tRNA) was entered, indicating that they provide no additional information. This indicates that plasma amino acid enrichment is the best determinant of amino acyl-tRNA enrichment. However, plasma KIC and tissue fluid amino acid enrichment are the most accurate predictors of tRNA enrichment.

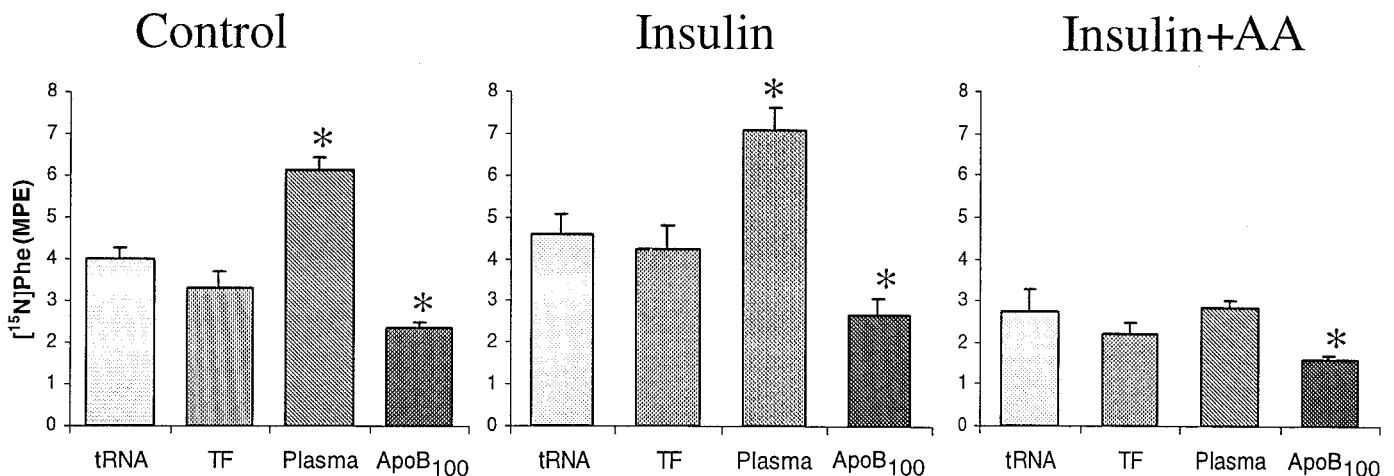


FIG. 2. Mean isotopic enrichment (molar percent excess [MPE]) of [¹⁵N]Phe enrichment (4–8 h) in tRNA, tissue fluid (TF), plasma, and ApoB₁₀₀. * $P < 0.05$ vs. tRNA.

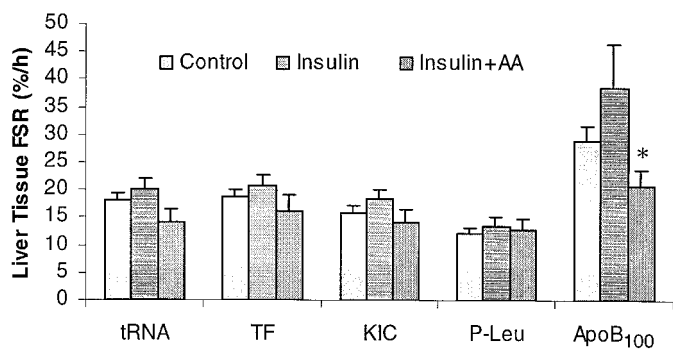


FIG. 3. Liver tissue protein FSR in three groups using enrichment values in [^{13}C]leucyl tRNA (tRNA), tissue fluid (TF), plasma [^{13}C]KIC, [^{13}C]Leu in plasma (P-Leu), and [^{13}C]Leu in ApoB₁₀₀ (ApoB₁₀₀) as precursor pools. *FSR using ApoB₁₀₀ is different in insulin + AA group from control and insulin groups ($P < 0.01$).

FSRs of liver proteins

Liver tissue protein synthesis. The FSR of liver tissue protein was not different among the three groups of pigs, except when ApoB₁₀₀ was used as a precursor pool. In that case, there was a decrease of liver tissue protein FSR in the insulin + AA group in comparison with the insulin group (Fig. 3).

Albumin synthesis rate. With the exception of ApoB₁₀₀, all other precursors led to estimations of lower albumin in the insulin group than in the control and insulin + AA groups. When ApoB₁₀₀ was used as a precursor, the insulin group was lower than the control group, but not lower than the insulin + AA group (Fig. 4).

Fibrinogen synthesis rate. The FSR of fibrinogen was lower in the insulin and insulin + AA groups than in the control group when tRNA, KIC, and tissue fluid Leu were used as precursors. When only plasma Leu was used as a precursor, the insulin group was lower than the control group, and when ApoB₁₀₀ was used, the insulin + AA group was lower than the control group (Fig. 5).

DISCUSSION

The current study allowed us to make important conclusions concerning the accuracy of the surrogate measures of tRNA and insulin's effect on liver protein synthesis. We compared the isotopic enrichments of [^{13}C]Leu and [^{15}N]Phe from various precursor pools, including plasma amino acids, ApoB₁₀₀, liver tissue tRNA, and tissue fluid under different physiological conditions in a swine model. It was demonstrated that isotopic enrichment of tissue fluid amino acid and plasma [^{13}C]KIC are the best predictors of

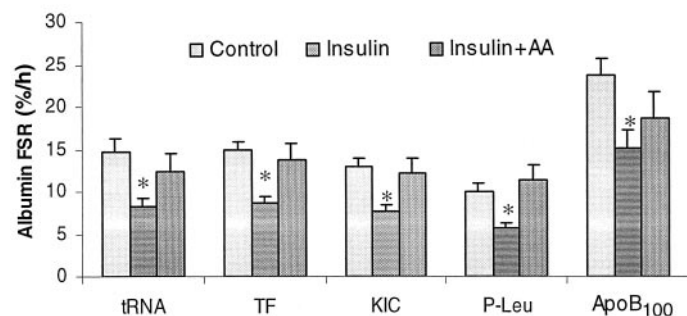


FIG. 4. Albumin FSR using L [^{13}C]Leu as a tracer in [^{13}C]leucyl tRNA (tRNA); tissue fluid (TF); plasma [^{13}C]KIC (KIC); [^{13}C]Leu in plasma (P-Leu); and [^{13}C]Leu in ApoB₁₀₀ (ApoB₁₀₀).

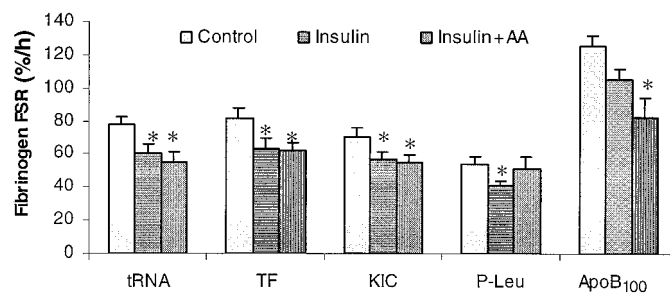


FIG. 5. Fibrinogen FSR using [^{13}C]Leu as a tracer. *Lower FSR in insulin and insulin + AA groups using [^{13}C]leucyl tRNA (tRNA), tissue fluid (TF), and plasma [^{13}C]KIC (KIC) as precursor pools ($P < 0.05$). Using [^{13}C]Leu in plasma (P-Leu) only in the insulin group and [^{13}C]Leu in ApoB₁₀₀ (ApoB₁₀₀) only in the insulin + AA group resulted in differences from the control group.

liver amino acyl-tRNA enrichment under different physiological conditions. In contrast, plasma amino acid enrichment values were substantially higher than those of tRNA, whereas VLDL ApoB₁₀₀ enrichment was lower than that of tRNA. The current studies were performed in animals during general anesthesia, which may have modulated some of the effects of insulin and amino acids, a factor that needs to be considered in extrapolating the data to normal physiology. However, similar study conditions were maintained during control study and interventions, and demonstrable but different effects of insulin and amino acids were observed.

We determined the effect of insulin, with or without amino acid supplementation, on the FSR of both liver tissue proteins and the liver-secreted proteins such as albumin and fibrinogen. The FSR of liver tissue proteins was unaffected by insulin or insulin plus amino acids. In contrast, fibrinogen synthesis was lower during both insulin infusion and insulin plus amino acid infusion. The albumin synthesis rate was reduced by insulin, but this inhibition effect of insulin was blunted by simultaneous administration of the amino acid mixture. The current study offered the opportunity to measure synthesis rates of liver proteins at two different insulin levels—baseline and elevated. In addition, the study offered the opportunity to determine the effect of insulin alone when circulating amino acids were low and when insulin and amino acid levels were both elevated. The latter condition is similar to a postprandial state after a high-protein mixed meal. The current study used liver amino acyl-tRNA as the precursor pool for measurement of protein synthesis and thus represents the most definitive information about insulin's effect on liver protein synthesis. The current study is in general agreement with the previous report.

It is generally accepted that the obligatory precursor of protein synthesis is amino acyl-tRNA within the tissue where protein is synthesized (35). However, it is not feasible to routinely sample liver tissue for measuring isotope enrichment of amino acyl-tRNA label in humans because of the logistical problems in collecting liver tissue for in vivo experiments and technical difficulties in measuring amino acyl-tRNA enrichment. In human studies, other accessible surrogate measures of amino acyl-tRNA have been extensively used. However, validation and comparison of isotope enrichments of these different precursors with that of tRNA have not been performed in order to provide an objective basis to predict the accuracy of

these different precursor pools as surrogate measures of tRNA. We performed the study in a mature swine model because of the similarity of swine to humans in several physiological and anatomical systems (36,37). Furthermore, it was possible to obtain simultaneous plasma and liver tissue samples and in larger amounts for testing the hypotheses. The measurement of various precursor pools for protein synthesis was possible because of the advances in mass spectrometry and innovation in other analytical techniques (34,38).

We demonstrated that the isotopic enrichment of tissue fluid or plasma KIC is the best predictor of tRNA when [^{13}C]Leu or [^{15}N]Phe are used as tracers. However, for Phe, there are no equivalents of KIC that can be measured in plasma. This finding supports the use of isotopic enrichment of plasma KIC (in the case of Leu) in lieu of enrichment of tRNA for calculations of liver protein FSR. It has been demonstrated previously that plasma KIC-specific activity better represents intracellular Leu-specific activity when radiolabeled Leu is infused in humans (39). In the current study, plasma [^{13}C]Leu enrichment, the most frequently used precursor pool, resulted in substantial underestimation of FSR and did not detect changes that occurred for FSR of fibrinogen during insulin and amino acid infusion. Similarly, calculation of the FSR of liver proteins using plasma [^{15}N]Phe may also lead to an underestimation of protein synthesis. This study also observed that the tracer enrichments in VLDL ApoB₁₀₀ are substantially lower than all other surrogate measures of precursor pool enrichment. As a result, assessment of liver protein synthesis using ApoB₁₀₀ surrogate measures of precursor pool labeling would substantially overestimate the FSR. Furthermore, using ApoB₁₀₀ enrichment for estimating protein synthesis does not lead to the same conclusions about insulin (with or without amino acid) as those reached using tRNA enrichment.

In a previous study, Baumann et al. (16) compared precursor enrichment of [^{13}C]Leu and [^{15}N]Phe in different tissues, including liver in a swine model in the baseline state. However, the comparison of isotope enrichment in VLDL ApoB₁₀₀ was not included in that study. Thus, the present study further extended previous results by including VLDL ApoB₁₀₀, which was reported as a true representation of liver amino acyl-tRNA precursor enrichment for estimating liver protein synthesis (19). The current study and that of Baumann et al. (16) found that tissue fluid isotope enrichment is similar to that of tRNA, indicating that there is a homogeneous pool at the site of protein synthesis within the cell. This conclusion is also strengthened by the observation that both tissue fluid [^{15}N]Phe and [^{13}C]Leu are identical to that of their respective tRNA enrichment values. The previous pig study (16) did not determine the impact of interventions such as insulin with or without amino acid infusion on the relationship of tRNA and other surrogate measures. Davis et al. (40) reported in neonatal piglets that insulin administration did not alter the relationship between amino acyl-tRNA and tissue fluid-specific activity. These studies enabled us to establish a stable relationship between tRNA enrichment and KIC enrichment under three physiological conditions—during the infusion of 1) saline, 2) insulin plus glucose, or 3) insulin plus glucose and amino acids. Overall, the

current study demonstrated that plasma [^{13}C]KIC enrichment is the most reliable predictor of tRNA enrichment in different study conditions.

An important finding of the present study is that the isotopic enrichment in VLDL ApoB₁₀₀ is much lower than that of tRNA. The lower enrichment of tracers in VLDL ApoB₁₀₀ in comparison with plasma amino acids and plasma has also been shown in other studies (5,41). VLDL ApoB₁₀₀ is synthesized exclusively in the liver with a rapid turnover rate. Until now, the isotopic enrichment in this protein has been claimed to represent the liver precursor pool labeling (19). However, there is no direct evidence that this surrogate precursor is an accurate measure of tRNA labeling in the liver. In the current study, we observed that [^{13}C]Leu enrichment in VLDL ApoB₁₀₀ is lower than that of both [^{13}C]KIC and tRNA. Also [^{15}N]Phe enrichments in VLDL ApoB₁₀₀ are less than that of tRNA. A previous study (42) reported that [^{13}C]Leu enrichment in VLDL ApoB₁₀₀ was 15% higher than in plasma [^{13}C]KIC. This observation is at variance with the findings of the present study, a difference that may be attributable to the different methodologies used for purifying VLDL ApoB₁₀₀ in plasma. It is likely that SDS-PAGE separation of VLDL ApoB₁₀₀, as performed in the present study, resulted in a purer separation, whereas the precipitation by isopropanol, as performed in the previous study (42), could not eliminate contaminations of other VLDL components that coprecipitated with ApoB₁₀₀. Contamination of labeled amino acid from extracellular fluid of plasma in amino acyl-tRNA was excluded by using a labeled internal standard (16) during tRNA isolation. No labeled standard was present in isolated amino acids from tRNA. We have used similar quality controls during mass spectrometry analyses of various precursors, and the differences among precursor pool enrichment was found to be unrelated to the small (<4%) interassay variations. The results from the present study, together with others (5,41), suggest that the measured liver protein FSR will be much higher if VLDL ApoB₁₀₀ is used as precursor enrichment. Furthermore, the estimation of protein synthesis based on ApoB₁₀₀ as a precursor pool does not truly reflect the changes in response to physiological changes as observed with the estimations using tRNA as a precursor pool.

To further support our conclusions that arterial plasma KIC is a valid and accurate measure of precursor pool labeling for measuring liver protein synthesis, we measured the FSR of liver tissue protein, albumin, and fibrinogen based on different precursors. As indicated in Figs. 3–5, the FSR of liver protein calculated using plasma KIC as a precursor gave a similar value as that using tRNA and tissue fluid, whereas the FSR calculated based on plasma [^{13}C]Leu or VLDL ApoB₁₀₀ gave results that were too low or too high, respectively. The lack of correlation between tissue protein FSR and albumin FSR reported by Barle et al. (4) was also confirmed in the present study.

Of note, our results indicated that insulin infusion in this pig model did not increase albumin synthesis. This may be accounted for by the fact that during insulin infusion, protein degradation is suppressed (26), which results in lower plasma amino acid concentrations, especially those of branched-chain amino acids (Table 2). Amino acid infusion along with insulin increased circulating insulin

and amino acids. This increased amino acid levels and maintained the albumin synthesis rate, supporting the importance of amino acids in maintaining the synthesis rate of albumin. We did not observe any stimulatory effect of amino acids on the albumin synthesis rate. It is known that insulin affects whole-body net protein anabolism by inhibiting protein breakdown (43,44). Amino acids are also known to decrease whole-body and muscle protein breakdown (45,46). Similar anabolic effects on albumin metabolism by amino acids or insulin remain to be investigated.

However, there are reports of albumin synthesis being stimulated in diabetic subjects when insulin was replaced (6) and being increased by enteral infusion of meals (7). During insulin deprivation, circulating amino acid levels are high, which may provide substrates for albumin synthesis in acute insulin replacement studies (6). These studies in type 1 diabetes have demonstrated that insulin is essential for albumin synthesis, a finding that is confirmed by studies on liver cultures (47). Results from the current study indicate that increasing insulin levels do not increase albumin synthesis, even when amino acid levels are high. These results are consistent with the normal albumin levels in hyperinsulinemic conditions such as obesity.

A differential effect of insulin and insulin plus amino acids on albumin and fibrinogen synthesis was also observed in the present study when fibrinogen synthesis was suppressed by the infusion of either insulin or insulin plus amino acids. This result is in agreement with that of De Feo et al. (6), in whose study human diabetic subjects showed decreased fibrinogen synthesis when insulin was infused. There was also no significant effect of insulin or insulin plus amino acid infusion on liver tissue protein synthesis. Insulin replacement in type 1 diabetic patients (27) and infusion of insulin in nondiabetic control subjects (26) have demonstrated that insulin decreases splanchnic protein synthesis. The current study supports the conclusion that neither insulin alone nor insulin plus amino acids has any stimulatory effect on mixed liver tissue protein synthesis, so that insulin's anabolic effect in liver seems to be exerted by mechanisms other than stimulation of protein synthesis. If insulin with or without amino acids increased liver protein synthesis and inhibited protein breakdown, one could expect expansion of liver size with frequent feeding. This phenomenon has not been described. Similarly, hyperinsulinemic states such as obesity also do not result in hepatomegaly. It is more likely that the liver maintains its remodeling processes (breakdown and synthesis) at a constant rate during postabsorptive and postprandial states. During the postprandial state, amino acids are provided by the meal, and during the postabsorptive state, they are provided by the muscle via a net increase in muscle protein breakdown (26).

In summary, the present study demonstrated that to assess liver protein synthesis with [¹³C]Leu as a tracer, when tissue biopsy is not available for direct measure of tRNA enrichment, the most accurate and convenient surrogate measure of precursor enrichment is [¹³C]KIC. Although amino acid enrichment of tissue fluid is another reliable surrogate measure of tRNA, the measurement of tissue fluid enrichment also requires liver biopsy, which is not feasible in most human studies. Plasma amino acid enrichment is much higher than that of tRNA, whereas the

amino acid enrichment in VLDL ApoB₁₀₀ is much lower than that of tRNA. Calculation of the liver protein FSR using either [¹³C]Leu in plasma or VLDL ApoB₁₀₀ would under- or overestimate the FSR, respectively. The current study also showed that the synthesis rate of liver tissue protein, albumin, and fibrinogen responds differently to insulin and amino acid infusions. Although insulin with or without amino acid infusion had no effect on mixed liver protein synthesis, there was a definite decrease in fibrinogen synthesis. Insulin alone reduced albumin synthesis, whereas supplementation of insulin with amino acids maintained the albumin synthesis rate, thus supporting the critical role of amino acids in maintaining albumin synthesis.

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