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Measurement of leucine and α-ketoisocaproic acid fluxes in the fetal/placental unit

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ABSTRACT

Many investigators are now using stable isotopes in place of radioactive isotopes because of ethical considerations in human research. Our laboratory has had a long history in the development of an ovine model for the study of the physiological and biochemical changes during pregnancy. We wanted to extend some of the hypotheses and experimental protocols developed while using this model system to the human. As a first step in this process, we carried out infusions using a mixture of both ^{14}C and ^{13}C isotopes of the essential amino acid L-leucine. Results from this study showed that turn-over rates calculated using the two isotopes were equal within experimental error (8.99 ± 0.45 and 8.97 ± 0.52 μmol/kg · min, respectively). A key step in the development of the techniques for this study involved the use of *tert.*-butyldimethylsilyl derivatives for the amino acids. Because of the strong M − 57 peak seen in the mass spectra of these compounds, we were able to use a relatively inexpensive Hewlett-Packard mass-selective detector for these determinations. Enrichments in triplicate measurements of the same sample had a precision of ±0.03%. Similar precision data were obtained in enrichment measurements of the keto acids derived from the amino acids. The combination of the speed of the analysis and the excellent precision have provided us with the opportunity to study uptake and loss across an organ system (*i.e.* placenta, liver, etc.). This tool is now being used to study the detailed flow of amino acids across the placenta during both normal and abnormal pregnancies.

INTRODUCTION

The detection and accurate measurement of a stable isotope in a biochemical matrix usually requires a mass spectrometer. The method of choice for amino acids has been the use of chemical ionization [1,2]. This method usually gives large molecular ions and facilitates the measurement of the enrichment of a single carbon in an amino acid. Other methods of ionization usually produce much more fragmentation or require a large sample preparation. Recently, it has been shown that the *tert.*-butyldimethylsilyl derivatives give a major fragment at M − 57 under electron-impact (EI) ionization. This has opened the door to the use of inexpensive, automated mass spectrometers [mass-selective detection (MSD)] to measure enrichment levels. In biochemical studies of the human, the amount of blood that one can draw is usually limited to 1 ml. If the study is on

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infants, the amount is even more restricted and this becomes even more complex if multiple samples must be taken (*e.g.* to determine a steady state).

We have recently reported on the development of a general technique for the rapid and precise measurement of amino acids and their corresponding keto acids from 0.2 ml of plasma [3]. The excellent precision attained when using this technique has provided us with an opportunity to measure uptake across organ systems [4]. In this paper we would like to present some of our recent data both from our animal and human studies.

EXPERIMENTAL

Sheep preparation

Laparotomy and catheterization of fetal and maternal blood vessel were performed on seven pregnant Columbia-Rambouillet sheep of ~130-day gestation after a two-day fast. Under pentobarbital sedation (6 mg/kg) and spinal anesthesia (6 mg tetracaine in 10% dextrose), polyvinyl catheters were placed in the fetal external iliac artery (α) via the dorsalis pedis artery, the fetal femoral vein via the saphenous vein (f), the common umbilical vein (γ), the maternal femoral artery (A), and the uterine vein (V) draining the pregnant horn. An additional catheter was placed in the amniotic cavity. The catheters were tunneled subcutaneously to the ewe's flank and protected in a plastic pouch secured to the ewe's skin. The animals were allowed to recover six or seven days with establishment of normal intake of *ad libitum* water and alfalfa pellets. Ampicillin (500 mg) was administered intramuscularly to the ewe and intra-amniotically on the first three postoperative days. In addition, streptomycin (500 mg) was given intramuscularly to the ewe at the time of surgery. At least two sheep were kept in the same room for company. The study was approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee.

On the day of study the fetus was infused via the femoral vein with a solution of L-[1-^{14}C]leucine ($6.2 \cdot 10^6$ dpm/ml) (ICN, Costa Mesa, CA, U.S.A.), L-[1-^{13}C]leucine (10.2 μmol/ml) (99%, MSD, Montreal, Canada) and antipyrine (0.1 g/ml) in normal saline. The rate of infusion was 0.079 ml/min. Antipyrine was included in the infusate for the purpose of measuring umbilical and uterine blood flows [5]. The infusion lasted 240 min. Samples from A, V, α and γ were drawn before the infusion and at 150, 180, 210 and 240 min during the infusion. The samples before infusion were used for blank measurements. Samples drawn during infusion were analyzed for antipyrine, oxygen saturation, oxygen capacity, hematocrit, blood glucose, plasma leucine, plasma α-ketoisocaproic acid (KIC), plasma leucine enrichment, plasma KIC enrichment, plasma [^{14}C]leucine and blood $^{14}CO_2$. Some of the samples were analyzed also for blood leucine and KIC. After the final draw, the infusion stopped, and simultaneously euthanasia solution (T-61, Taylor Pharmaceutical, Decatur, IL, U.S.A.) was injected into the maternal and fetal circulations. The fetus and placental cotyledons were re-

moved, weighed and homogenized separately. The homogenates were stored at −70°C and subsequently analyzed for total ^{14}C and [^{14}C]leucine content.

Analytical methods

Antipyrine was measured by an automated version [5] of the method of Brodie *et al.* Blood hemoglobin concentration, expressed as oxygen capacity, and oxyhemoglobin saturation were measured in duplicate in a spectrophotometer (OSM-2, Radiometer, Copenhagen, Denmark). Blood oxygen content was calculated as the oxygen capacity times oxygen saturation product. Whole blood glucose was measured by a glucose oxidase method (Sigma, St. Louis, MO, U.S.A.) in a protein-free filtrate. The leucine and [^{14}C] leucine content of plasma, blood and tissue samples were analyzed according to the procedure that has been described previously [7]. In this procedure, plasma and blood samples are deproteinized with sulfosalicyclic acid and analyzed for leucine by means of a Jeol-200A amino acid analyzer. Trace leucine is measured separately on fractions collected from an ion-exchange column packed with LCR-2 resin and eluted with sodium citrate buffer (pH 4.25) at 41°C. The tissue samples are hydrolyzed, dried and redissolved in buffer prior to analysis. Blood $^{14}CO_2$ was measured on 0.3-ml samples, as described by Van Veen *et al.* [7].

The [1-^{13}C]leucine and KIC enrichments and KIC concentrations were measured as described in detail elsewhere [3]. Briefly, 0.2 ml of plasma were acidified and mixed with a solution containing ketovaleric acid as internal standard of the KIC concentration. The amino acids and keto acids in the plasma–internal standard mixture were separated via an ion-exchange resin column procedure and derivatized. The *tert.*-butyldimethylsilyl derivatives were formed for amino acids and the trimethylsilyl quinoxalinol derivatives were formed for keto acids. The derivatized leucine and KIC samples were analyzed as separate runs by means of a HP-5890 gas chromatograph coupled with a HP-5970 mass spectrometer.

RESULTS AND DISCUSSION

The development of an analytical technique that is both convenient and precise has opened the way for a series of studies of the utilization and mass movement (flux) of amino acids across the placenta. This technique has been shown to be applicable to a series of amino acids [3] and their corresponding keto acids. Furthermore, the technique was validated using both ^{13}C and ^{14}C labels by simultaneous infusion into seven animals. The overall disposal rate (fetus + placenta) was shown to be the same for both, 8.98 ± 0.48 μmol/kg · min.

The use of an EI ion source and an autoinjector have both been important to this research. Mass spectrometers using standard EI are relatively inexpensive, easy to maintain (when compared to chemical ionization) and give reproducible results over the course of the more than 100 injections required for each of our studies. The autoinjector allows one to complete each study after only two to

three days of continuous use. Use of a gas chromatographic–mass spectrometric system provides a level of specificity that is difficult to achieve in other analytical systems.

The infusion of a substrate such as leucine into the fetal compartment provides interesting new data about the rate of use and the rate of appearance of unlabelled leucine by the fetus and the placenta. Examination of Fig. 1 shows that after about 2 h of infusion the fetus has reached a steady state. The enrichment in the fetal artery (α) and the rate of infusion can be used to determine the disposal rate of leucine in the animal [6]. The reduction in enrichment between the artery entering the placenta (α) and the vein draining the placenta (γ) indicates that unenriched leucine has entered the fetal circulation. This is very likely to be new, maternal leucine entering the fetal circulation. Furthermore, the lack of enrichment seen in the maternal vein (V) would suggest that leucine is not allowed to cross the placenta from the fetus to the mother. In the case of leucine, the placenta supplies amino acid from the mother's circulation but prohibits its return.

An infusion of the same L-leucine into the maternal circulation results in a steady state as shown in Fig. 2. The highest levels of enrichment are seen in the maternal artery (A). Inspection of the fetal circulation shows that the enriched isotope is transferred across the placenta and into the fetal circulation. The facts that the fetal vein leaving the placenta (γ) has more enrichment than the fetal artery confirms that new leucine is entering the fetal circulation from the mother.

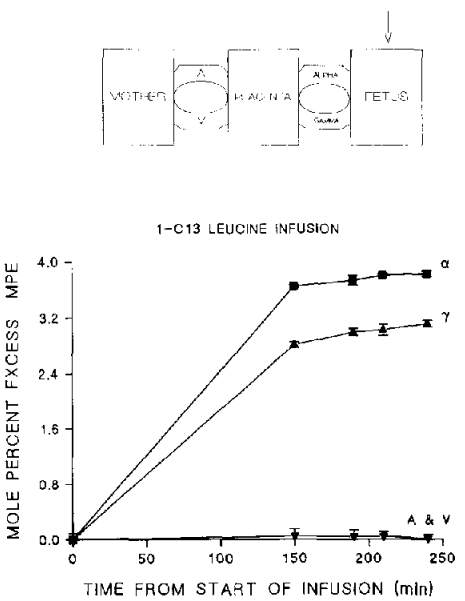

Fig. 1. Results of infusion of [1-^{13}C]leucine into the fetal circulation showing a dilution in the isotope as blood flows across the placenta. No measurable enrichment is seen in maternal circulation (A, V).

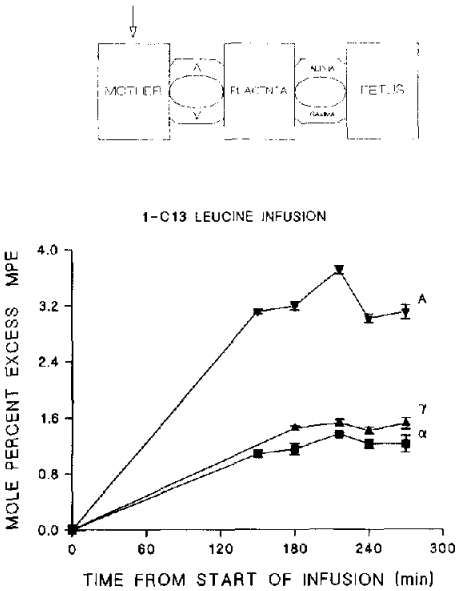

Fig. 2. Results of infusion of [1-^{13}C]leucine into the maternal circulation. Approximately 40% of the maternal enrichment (A) is seen in the fetal circulation (α, γ) indicating dilution of enriched isotope in the placenta and fetus. The higher enrichment in γ indicates net influx of leucine from the maternal circulation via the placenta.

A combination of these experiments provide a means of estimating the flux of amino acid entry from the mother.

This apparently straightforward experiment is made more complex by the rapid interconversion of many amino acids into their corresponding keto acids. In the case of leucine in the fetal sheep, it was found that 66% of the plasma keto acid was labelled. Studies in the human neonate revealed a 87% enrichment for this same keto acid. Because this is a rapid equilibrium the entry rate calculated above, when only leucine enrichments are considered, results in a low estimate of the true flux. A more accurate estimate of the true flux can be determined by measuring the enrichment and steady state values of both leucine and KIC. The sum of the fluxes of these two will better estimate the net flow of amino acid into the fetus.

A combination of the data described above with the oxidation data provided by measuring enrichment in CO_2 has led to the formulation of the flux balance diagram shown in Fig. 3. In this model approximately one half of the leucine in the fetal circulation is committed to development (accretion of protein) and the other half is either oxidized or transferred to the placenta for additional metabolism. The residual entries (shown in Fig. 3) in both the fetus and placenta represent unaccounted enrichment (error) in our calculation. This 10% error is rather small when one considers the complexity of the *in vivo* model we are using. This

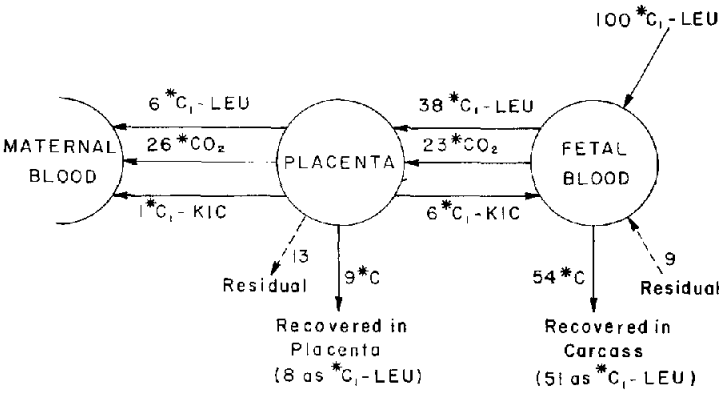

Fig. 3. Relative disposition of [1-^{13}C]leucine infusion into fetal circulation. The amount infused is given as the arbitrary amount of 100 and all other numbers are relative to that value.

type of a balance can be used to help us understand the complexity of the system and to suggest new ways of testing and refining the data base. Additional studies using other amino acids are currently underway.

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