Tracer Kinetics Are of Limited Value to Measure In Vivo Protein Synthesis and Degradation Rates in Muscle of Anesthetized Rats

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Measurement of amino acid kinetics using muscle exchange rates of labeled phenylalanine and leucine has been successfully used to estimate in vivo protein synthesis and degradation rates in human forearm and in hindlimb of large laboratory animals. No good method to measure protein breakdown in muscle of small laboratory animals is available, and we therefore investigated whether this technique can be applied to rats. Using [^{3}H]phenylalanine-exchange measurements, protein synthesis and degradation rates were measured in muscle of fed and 2-day starved rats. Protein synthesis rates obtained in this way were compared with rates measured with the phenylalanine flooding-dose technique in sham-cannulated (including anesthesia and surgery) fed and fasted rats and in awake fed rats. Using the [^{3}H]phenylalanine-exchange method, protein synthesis rates in 2-day starved rats were increased to 292% and protein degradation rates to 217% of the values obtained in fed rats. However, due to a high variation, these changes were not statistically significant. Results obtained with the flooding-dose technique indicate that 2-day starvation reduced protein synthesis rates to 61% of the fed value. However, protein synthesis rates measured with the flooding-dose technique were decreased by 40% in sham-cannulated fed rats in comparison to awake fed rats. An additional 19% reduction in adenosine triphosphate (ATP) concentration in the muscle of the same rats shows that the procedure necessary to apply the exchange measurements to rats has a significant influence on the physiology of the muscle. We therefore conclude that [^{3}H]phenylalanine-exchange measurements as applied in this study are of limited value to estimate in vivo protein synthesis and degradation rates of individual tissues in rats.

MUSCLE PROTEIN WASTING is a common feature observed in many diseases, and has repeatedly been reported to be a major complication in patients with trauma and sepsis. To investigate the mechanism of muscle wasting, preferably both protein synthesis and protein degradation should be measured in vivo. For measuring protein synthesis alone, determining the incorporation of labeled amino acids in tissue protein following a primed-continuous infusion or a flooding dose are generally accepted methods.[1,2] However, few techniques are available to estimate in vivo protein degradation rates.[1-3] Of these, the exchange rates of leucine and phenylalanine tracers across individual tissues seem the most reliable for estimating both protein synthesis and degradation in large animals (dogs and lambs) and in humans.[4-11] Many animal models for studying metabolic derangements during disease states have been developed in rats, and have created a need for a method to measure protein degradation rates in small animals. Sugden and Fuller[2] suggested potential technical problems in small animals with cannulation and plasma flow measurements necessary to apply the tracer-exchange method. Both of these techniques have been adapted for use in rats.[12] In this study, we therefore investigated whether [^{3}H]phenylalanine-exchange measurements can be used in rats to simultaneously estimate tissue rates of protein degradation and synthesis in muscle.

Using [^{3}H]phenylalanine exchanges across hindquarter, we estimated protein synthesis and degradation rates in muscle of fed and 2-day starved rats. Protein synthesis rates obtained in this way were compared with rates obtained with the flooding-dose technique applied under the same circumstances (sham cannulation, including anesthesia and surgery) and in nonoperated fed rats. Five groups of rats were studied: two fed and two fasted groups for measuring protein synthesis with one of two techniques ([^{3}H]phenylalanine exchange or flooding dose) under anesthesia and surgery (needed to perform the exchange measurements), and one fed group for measuring protein synthesis with the flooding-dose technique in the awake fed control animal. In addition, concentrations of high-energy phosphates were measured in muscle of sham-cannulated and awake fed rats to check the physiological state of the muscle during the experimental procedure.

MATERIALS AND METHODS

Principle of the [^{3}H]phenylalanine-Exchange Measurements

For an amino acid with a constant intracellular concentration, the net uptake or release is the difference between production and utilization.[4] For phenylalanine, which is neither synthesized nor degraded in skeletal muscle, at steady state the net balance across muscle tissue is the difference between protein synthesis and degradation,[8] and therefore

$$\text{release} = \text{uptake} - \text{net balance, and} \qquad \text{Eq 1}$$

$$\text{protein degradation} = \text{protein synthesis} - \text{net balance.}$$

According to the Fick principle, net balances can be calculated from the arteriovenous difference for phenylalanine and the plasma flow with the equation,

$$\text{net balance} = ([\text{Phe}]_a - [\text{Phe}]_v) \times \text{flow}, \qquad \text{Eq 2}$$

where $[\text{Phe}]_a$ and $[\text{Phe}]_v$ are the phenylalanine concentrations in the artery and the vein. Protein synthesis can be estimated by measuring net uptake of vein. phenylalanine from the artery:

$$\text{protein synthesis} = \text{ER} \times [\text{Phe}]_a \times \text{flow}. \qquad \text{Eq 3}$$

The extraction rate (ER) for labeled phenylalanine is the arteriovenous difference in radioactivity divided by the arterial radioactivity (all in disintegrations per minute per milliliter).

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The rate of protein degradation across the hindquarter is estimated from the dilution of the specific activity (SA) of phenylalanine by unlabeled phenylalanine released from tissue protein due to protein degradation. This can be calculated from equations 1 to 3:

$$\text{protein degradation} = (ER \times [Phe]_a \times \text{flow}) - \{([Phe]_a - [Phe]_v) \times \text{flow}\} = \text{flow} \times [Phe]_v \times (1 - SA_v/SA_a), \quad \text{Eq 4}$$

in which SA is the SA of phenylalanine (disintegrations per minute per nanomole).

These calculations do not take into account the reutilization of phenylalanine derived from protein breakdown, and therefore synthesis and degradation rates are minimal estimations.

These assumptions and calculations are valid only in a steady state during isotopic equilibrium of L-[4-^{3}H]phenylalanine in plasma. A primed-constant infusion with a priming bolus of 0.55 μCi (18 pmol)/100 g body weight followed by an infusion of 0.44 μCi (15 pmol)/100 g body weight/h results in a plateau within 30 minutes in rats weighing 250 to 300 g (Fig 1).

Since no differences in phenylalanine uptake and release have been observed using either plasma or whole-blood values,[8] plasma was used in the present study.

Experiments

All experiments were performed using male Wistar rats (250 to 300 g; Winkelmann, Borchen, Germany) individually housed under standard conditions (12-hour light-dark cycle) and fed standard laboratory chow and water ad libitum. On day 0, rats were randomly assigned to one of the following groups (5 to 6 rats per group): (A) fed, [^{3}H]phenylalanine-exchange measurement; (B) 2-day fasted, [^{3}H]phenylalanine-exchange measurement; (C) fed, [^{3}H]phenylalanine flooding-dose technique, sham-cannulation; (D) 2-day fasted, [^{3}H]phenylalanine flooding-dose technique, sham-cannulation; and (E) fed, [^{3}H]phenylalanine flooding-dose technique, nonrestrained awake. After a 4-day acclimatization period, food was taken away from rats in the fasted groups (B and D) in the morning. Experiments were performed 48 to 54 hours later. Food was taken away from fed rats just before the start of measurements, and these rats are therefore fasted for 0 to 6 hours.

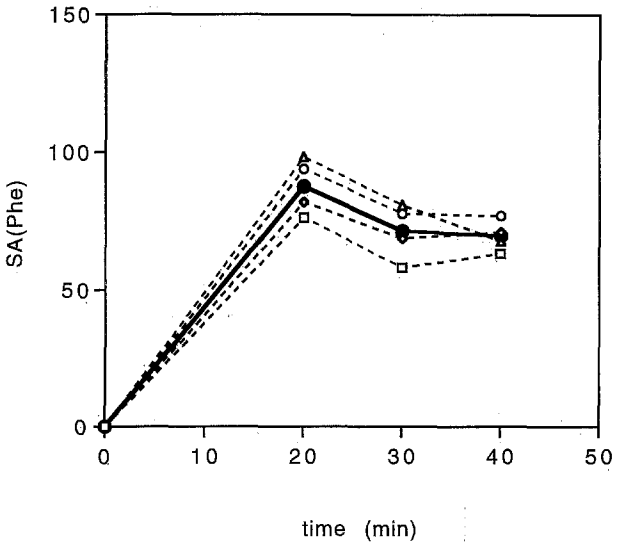

Fig 1. SA (dpm/nmol) of [^{3}H]phenylalanine during a primed-continuous infusion of L-[4-^{3}H]phenylalanine (0.55 μCi and 18 pmol/100 g body weight as a bolus, followed by a continuous infusion of 0.44 μCi and 15 pmol/100 g body weight/h) into a jugular vein catheter. Approximately 400 μL blood was sampled from a carotid artery catheter just before starting the infusion and at 20, 30, and 40 minutes during the infusion in 4 anesthetized rats. Values at 30 and 40 minutes were not significantly different (Mann-Whitney *U* test, *P* = .564). (□) 1, (◇) 2, (○) 3, (△) 4, (●) mean.

Groups A and B. Fed and 2-day starved rats were anesthetized using ether. Rectal temperature was monitored and maintained at or close to preanesthesia levels using a heating blanket. The right carotid artery and left jugular vein were cannulated with PE-50 catheters. The inferior caval vein and the abdominal aorta (just above the bifurcation) were cannulated with a 25-gauge needle fitted to silastic tubing (Silastic Medical Grade Tubing, 0.051 cm ID, 0.094 cm OD; Dow Corning, Midland, MI) and cemented in place with cyanoacrylate adhesive. After positioning the catheters, L-[4-^{3}H]phenylalanine was infused (0.55 μCi and 18 pmol/100 g body weight as a bolus, followed by a continuous infusion of 0.44 μCi and 15 pmol/100 g body weight/h) into the jugular vein until a steady state was reached (30 minutes). For blood flow determination, *p*-aminohippuric acid ([PAH] 5 mmol/L, iso-osmotic, pH 7.4) infusion into the aortic catheter was started 10 minutes after positioning the catheters. A priming dose of 0.15 mL/100 g body weight of a 50-mmol/L PAH solution was followed by continuous infusion of a 5-mmol/L PAH solution at a rate of 1.5 mL/h.[12] Approximately 30 minutes after [^{3}H]phenylalanine infusion was started, blood samples were obtained by slowly aspirating blood (±800 μL), first from the carotid artery and immediately thereafter from the inferior caval vein. Blood samples were kept on ice in heparinized cups until further processing (maximal, 3 minutes). Part of the blood sample was deproteinized with perchloric acid for PAH measurements. Of the remainder, plasma was separated by centrifugation and used for determination of phenylalanine SA. Plasma (100 μL) was deproteinized with sulfosalicylic acid for amino acid analysis. Processed samples were stored at −80°C until analysis.

Groups C and D. Fed and 2-day starved rats were sham-cannulated (including anesthesia and surgery) as described for groups A and B. Ten minutes after positioning the catheters, an infusion of PAH (as above) into the aortic catheter was started. Five minutes later, a flooding dose of L-[4-^{3}H]phenylalanine (150-mmol/L, 40 μCi/mL, 1 mL/100 g body weight) was injected into the jugular vein as described previously.[13] Muscle (gastrocnemius) was sampled 15 minutes later, rapidly frozen in liquid nitrogen using a precooled pair of tongs, and stored at −80°C until analysis of [^{3}H]Phe incorporation into protein. Due to the laparotomy necessary to insert the catheters, the flooding dose had to be administered intravenously instead of intraperitoneally. No difference versus results obtained with intraperitoneal administration is to be expected, because comparable fractional synthesis rates were obtained with both techniques by Garlick et al[13] and Jepson et al.[14]

Group E. Fed control rats were administered an intraperitoneal injection of a flooding dose of L-[4-^{3}H]phenylalanine (150-mmol/L, 20 μCi/mL, 2 mL/100 g body weight) as described previously.[14] The rats were killed by cervical dislocation 15 minutes later, and muscle (gastrocnemius) was rapidly sampled, frozen in liquid nitrogen using a precooled pair of tongs, and stored at −80°C until analysis.

Measurements

Plasma amino acid concentrations were determined by high-performance liquid chromatography.[15] For PAH determination in whole blood, we used a modification[12] of the method used by Brun.[16] Plasma PAH concentration was calculated from whole-

blood PAH concentrations using the hematocrit. Plasma flow was calculated as follows[12,17]:

$$\text{flow} = \frac{I}{[PAH]_v - [PAH]_a} \times \frac{100}{\text{body weight}} \cdot [\text{mL} \cdot \text{min}^{-1} \cdot 100\ \text{g body weight}^{-1}]. \quad \text{Eq 5}$$

I is the infusion rate of PAH (micromoles per minute), and $[PAH]_v$ and $[PAH]_a$ are PAH concentrations in venous (v) and arterial (a) blood. Since plasma flows were not available for all animals, the mean flow per group was used for calculation instead of individual flows.

Phenylalanine SA (disintegrations per minute per nanomole) in the deproteinized plasma was determined using a modification[13] of the method described by Suzuki and Yagi.[18] Phenylalanine was converted to β-phenylethylamine with tyrosine decarboxylase and pyridoxal phosphate and extracted using chloroform/heptane. Both the concentration and radioactivity were determined to calculate SA:

SA = radioactivity (dpm/mL)/concentration (nmol/mL). Eq 6

Concentrations of adenosine triphosphate (ATP), diphosphate (ADP), and monophosphate (AMP), creatine phosphate, and creatine were measured in perchloric acid (1 mol/L) extracts of muscle tissue from groups C and E using enzymatic methods.[19] These methods were adapted for a spectrophotometric centrifugal analyzer (COBAS-BIO; Roche Diagnostics, Montclair, NJ).

Calculations

For calculating net balance and protein synthesis and degradation rates, the equations described earlier were used. When using the flooding-dose technique, fractional protein synthesis rates (FSRs) were calculated from phenylalanine SA in the intracellular amino acid pool (precursor pool) and protein-bound phenylalanine SA in muscle[13,14]:

$$\text{FSR} = \frac{SA_{bound}}{SA_{precursor}} \times \frac{1}{\text{time}} \times 100[\%/\text{d}]. \quad \text{Eq 7}$$

For the intraperitoneally administered flooding dose as used for group E, SA of the precursor pool was multiplied by 0.9 to correct for the longer period required to reach isotopic equilibrium in the intracellular pool, as suggested previously.[14]

Statistical analysis of differences between groups was performed using the nonparametric Mann-Whitney *U* test. Significance was set at *P* less than .05.

RESULTS

Body weight was significantly decreased in 2-day starved rats compared with fed control (258 g [range, 242 to 266] *v* 304 g [range, 292 to 314]). Hindquarter plasma flow rates were not different between fasted and fed rats (mean ± SEM: 1.92 ± 0.54 *v* 2.11 ± 0.68 mL · min^{-1} · 100 g body weight^{-1}).

Using [^{3}H]phenylalanine-exchange measurements, protein synthesis rates in 2-day starved rats were increased to 292% and protein degradation rates to 213% of the fed values (Table 1). Due to the high variation, these changes were not statistically significant. The net balance of phenylalanine across muscle was not different from zero in both fed and 2-day starved rats (Table 1). Protein synthesis rates measured with the flooding-dose technique in sham-cannulated rats were decreased in starved rats to 61% of the value obtained in fed rats (Table 1). Protein synthesis rates measured in muscle of awake fed rats were significantly higher (65%) than in anesthetized sham-cannulated fed rats (Table 1).

Table 1. Protein Synthesis and Degradation Rates

Parameter	Fasted	Fed
Groups A and B		
Net balance (nmol Phe/min/100 g BW)	−10.8 (−24.9-10.9)	−7.5 (−36.1-9.5)
Degradation (nmol Phe/min/100 g BW)	49.3 (16.9-73.9)	22.7 (13.6-32.7)
Synthesis (nmol Phe/min/100 g BW)	38.4 (3.7-84.8)	15.2 (−8.4-30.3)
Groups C and D		
Synthesis (%/d)	4.6 (3.6-5.2)*	7.5 (5.5-9.4)
Group E		
Synthesis (%/d)		12.4† (9.2-20.9)

NOTE. Values are the mean (range) for 5 to 6 rats.
Abbreviation: BW, body weight.
*Significantly different from the fed group.
†Significantly different from the fed, sham-cannulated, flooding-dose group.

Concentrations of ATP and ADP were significantly decreased in muscle of sham-cannulated fed rats in comparison to awake fed rats. No differences were observed in concentrations of AMP, creatine phosphate, and the sum of creatine and creatine phosphate (Table 2).

DISCUSSION

Changes in muscle protein content are the result of the net balance between protein synthesis and degradation rates, and, therefore, preferably both should be measured when investigating mechanisms of protein deposition or wasting. In vivo estimations of both protein synthesis and degradation rates obtained with exchange measurements using amino acid tracers have been successfully used in human subjects and large laboratory animals.[4-11] Thompson et al[20] have used the technique to measure protein turnover in human forearm. The protein synthesis rate they obtained was in the same quantitative range as rates obtained with other techniques, and they concluded that phenylalanine-tracer kinetics are therefore valid to measure protein turnover in human muscle. Barret et al[8] concluded that the technique can be used for estimating in vivo protein turnover in specific muscle beds of anesthetized dogs. In small laboratory animals such as rats and mice, incorporation of labeled amino acids is a generally accepted measure

Table 2. Concentrations (μmol/g wet weight) of Intracellular Muscle Metabolites

Metabolite	Sham-Cannulated	Control
ATP	5.23* (4.36-6.26)	6.45 (6.14-6.77)
ADP	0.67* (0.53-0.78)	0.83 (0.75-0.95)
AMP	0.09 (0.07-0.14)	0.07 (0.05-0.08)
Creatine phosphate	17.7 (14.5-22.2)	17.7 (13.4-19.8)
Creatine + creatine phosphate	27.6 (23.1-35.5)	32.0 (29.8-33.1)

NOTE. Values are the mean (range) for 5 rats.
*Significantly different from the control group.

of in vivo protein synthesis rates, but no good method is available to measure protein degradation rates in vivo.[1,2] Sugden and Fuller[2] suggested that tracer-exchange measurements may be readily applicable only to larger animals and human subjects, because of potential technical problems with cannulation and plasma flow measurements in small animals. Both of these techniques have been adapted for use in rats.[12] We investigated herein whether L-[4-^{3}H]phenylalanine-exchange measurements can be used to estimate in vivo protein synthesis and degradation rates in rat skeletal muscle.

Using [^{3}H]phenylalanine exchanges, mean protein synthesis rates were almost threefold higher in fasted rats than in fed rats. However, this increase was not statistically significant, due to a large variation between individual animals. Also, net balance and protein degradation values showed a high variation. Power analysis showed that greater than 75 rats per group are needed to measure a statistically significant difference in the order of the starvation-induced decrease in protein synthesis measured with the flooding dose.

Starvation resulted in a reduction of protein synthesis rates when measured with the flooding-dose technique in sham-operated and cannulated rats. The effect of starvation on protein synthesis rates in rat muscle has been measured with different techniques: primed-constant infusion in vivo,[21] incubated muscle,[22,23] perfused hindquarter,[24] and in vivo flooding dose.[14,25-27] In all of these studies, starvation has been reported to cause a reduction in protein synthesis rates. Cheng et al,[7] using labeled-phenylalanine–exchange measurements, observed decreased protein synthesis rates in the forearm muscle of fasted human subjects. The threefold increase in apparent protein synthesis rates calculated herein from [^{3}H]phenylalanine-exchange measurements in 2-day starved rats is in contradiction to these findings. Although the flooding-dose technique is controversial in human studies, in animal studies it is generally in good agreement with other methods.[28] The advantage of this technique in rats is that no anesthesia and cannulation are needed, which could interfere with the measurements.

The large variation in the exchange measurements could be the result of the high variation in blood flow measurements or a fluctuation in the physiological state of the rat. Calculations of protein synthesis and degradation rates using [^{3}H]phenylalanine-exchange measurements were based on only one sampling point in this study, because a greater volume of blood cannot be obtained from rats without the risk of hypovolemia. Protein synthesis rates measured with the flooding-dose technique were mean values of a 15-minute incorporation period, and, therefore, physiological variation in time due to depth of anesthesia, for instance, will have a larger influence on values obtained with the exchange measurements. Also, variations in muscle metabolite concentrations were larger in the sham-cannulated group than in awake rats, indicating a larger variation in the physiological state of cannulated rats. Most likely, the variation due to variability in blood flow can be improved by identifying the source of the error. However, the decreased protein synthesis rates and ATP and ADP concentrations in muscle of sham-cannulated fed rats in comparison to awake fed rats show that the procedure necessary to apply the measurements has a significant influence on the physiological state of the rat. Ether anesthesia has been reported not to influence or to only slightly influence protein synthesis rates and concentrations of energy substrates in skeletal muscle of rats.[29,30] This suggests that, most likely, the combination of anesthesia, surgery, catherization, and sampling influence the physiological state of the rat. We therefore conclude that [^{3}H]phenylalanine-tracer kinetics as performed in this study do not reliably estimate in vivo protein turnover rates of rat muscle.

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