Skeletal muscle and whole-body protein turnover in cirrhosis

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SUMMARY

1. We investigated arteriovenous exchanges of tyrosine and 3-methylhistidine across leg tissue in the postabsorptive state as specific indices of net protein balance and myofibrillar protein breakdown, respectively, in eight patients with cirrhosis and in 11 healthy control subjects. Whole-body protein turnover was also measured using \( L-\[^{1-13}C\] \) leucine.

2. Leg efflux of tyrosine was 45% greater in cirrhotic patients than in normal control subjects \((-6.5 (1.4 to -19.1) \text{ vs } -4.2 (-2.2 to -7.7) \mu \text{mol min}^{-1} 100 \text{ mg}^{-1} \text{ of leg, median (range), } P < 0.025\)\. 3-Methylhistidine efflux was not significantly altered.

3. In cirrhosis, whole-body leucine flux was normal but whole-body leucine oxidation was elevated so that whole-body protein synthesis was depressed by 17%.

4. The results indicate the predominant mechanism of muscle wasting in cirrhosis to be a fall in muscle protein synthesis, which is accompanied by an overall fall in whole-body protein turnover.

Key words: amino acids, cirrhosis, leg muscle, protein turnover, stable-isotope tracers.

Abbreviations: BCAA, branched-chain amino acids; \( \alpha-KIC \), \( \alpha \)-ketoisocaproate; \( S_0 \), oxygen saturation; \( V\text{CO}_2 \), rate of \( \text{CO}_2 \) production.

INTRODUCTION

Cirrhosis may be accompanied by muscle wasting and myopathy [1], which could be due to a reduction in protein synthesis, an increase in breakdown, or some combination of the two [2]. Previous workers have claimed, on the basis of urinary 3-methylhistidine excretion, that muscle wasting in cirrhosis is chiefly due to accelerated protein breakdown [3, 4], but such an interpretation of the experimental observations has been contested on the grounds that small but rapidly turning over pools of 3-methylhistidine exist in, for example, smooth muscle which may contribute substantially to whole-body (urinary) 3-methylhistidine [5]. The aim of the present study was to investigate the mechanism of muscle wasting in cirrhosis by studying arteriovenous amino acid exchanges across leg tissue (which is 70-80% skeletal muscle) and also whole-body protein turnover using stable-isotope-labelling techniques, and comparing the results with those from normal control subjects.

In order to gain an insight into muscle protein metabolism, we adopted a relatively simple technique which involves measuring production by limbs of the amino acids tyrosine and 3-methylhistidine. 3-Methylhistidine in myofibrillar protein is the result of methylation of actin and myosin [6, 7]; after their breakdown, it cannot be re-utilized for protein synthesis and its efflux from muscle is therefore an index of myofibrillar protein breakdown [8, 9]. In venous samples drawn from a major vein draining a limb (such as the femoral vein) the contribution of non-skeletal muscle sources of 3-methylhistidine (e.g. gut or lung) are minimized, thus obviating the problems associated with interpretation of urinary production. Previous studies have shown that the arteriovenous balance of tyrosine, an amino acid which is not catabolized in skeletal muscle [10], usually correlates very closely with the total amino acid arteriovenous balance [11, 12]. By knowledge of the net protein balance, and with some index of breakdown, the extent of protein synthesis can be estimated semi-quantitatively.

Whole-body protein turnover was measured by stable-isotope methodology [13], using a primed constant infusion of \( L-[^{13}C] \) leucine. At plateau-labelling of leucine in the free amino acid pool (achieved within 2 h) the extent of the labelling gives an indication of the flux of tracer through that pool; the processes of protein synthesis or amino acid oxidation effectively draw upon a precursor pool in a steady state with regard to labelling.
The model used for leucine metabolism generates the equation [13]:

\[ Q = S + \dot{O} = B + D \]

where \( Q \) is the rate of leucine turnover (or flux), \( S \) is the rate of protein synthesis, \( \dot{O} \) is the rate of leucine oxidation, \( B \) is the rate of amino acid derived from protein breakdown and \( D \) is the dietary intake from food (which is zero since the patients were studied postabsorptively). All rates are expressed as \( \mu \text{mol of leucine h}^{-1} \text{kg}^{-1} \). Leucine turnover (flux) through free amino acid pool can be calculated by the dilution of the \( l-[\text{1}^{13}\text{C}] \) leucine infused into the plasma leucine pool, once steady-state conditions are reached. However, leucine is metabolized first by transamination to \( \alpha \)-ketoisocaproate (\( \alpha \)-KIC) [14] and then by decarboxylation to isovaleryl-coenzyme A and \( \text{CO}_2 \), and it has become accepted that a better indication of whole-body leucine flux (i.e. both extra- and intracellular) can be obtained by relating the infusion rate of leucine tracer to the labelling of the major metabolite \( \alpha \)-KIC [15]. There is no evidence that the extent of leucine and KIC exchange is different in cirrhotic patients and normal subjects, and the evidence from studies in man suggests that muscle transaminase activity is sufficient to rapidly equilibrate the pools. Whole-body leucine oxidation may be measured by means of collection of expired \( \text{CO}_2 \) with subsequent cryogenic purification and measurement of \( ^{13}\text{CO}_2 \) enrichment by isotope ratio mass spectrometry [16]. By measuring flux the rate of leucine release from protein (i.e. breakdown) is obtained directly, and from this, if the rate of oxidation is known, the rate of leucine incorporation into protein (i.e. synthesis) can be calculated.

**METHODS**

**Patients**

Eight patients (two women and six men) with chronic liver disease (seven with alcoholic liver disease and one with primary biliary cirrhosis), mean age 59.1 years (range 51–70 years), were compared with 11 healthy normal control subjects (two women and nine men), mean age 45 years (range 23–70 years) (for details see Table 1). We chose to compare results from the patients with a single large wide-age-range control group. In fact, the conclusions would have been no different if we had used a sub-division of the control group matched to the patient group, since we found no significant age-related effects on limb or whole-body amino acid metabolism. Four of the patients had histological evidence of cirrhosis on liver biopsy; the remaining four could not be biopsied because of coagulopathy, but of these three have since died and had histological evidence of cirrhosis post mortem. All patients had oesophageal varices; six had a previous history of an episode of liver failure and two had a history of ascites. Patients were studied when they were in as fit a condition as possible, five after a period of inpatient treatment. All patients were losing weight at the time of study. None of the patients had drunk alcohol for 1 month before the study, and two had clinically detectable ascites and mild peripheral oedema at the time of study. No patients were encephalopathic at the time of study and none had ketonaemia.

**Investigative procedures**

All studies commenced at 08.00 hours in a ward side-room, with the patient in bed, having not eaten or drunk for 16 h before the study. The purpose and nature of the investigation was explained to all subjects and written consent was obtained. The investigation had the approval of the Tayside Health Board and Medical School Ethical Committees. A superficial dorsal hand vein was cannulated retrogradely using a 16G cannula (Venflon; Viggo Ltd) attached to a three-way tap to enable blood sampling. The cannulated hand was placed in a thermostatically controlled hot box at 65°C which effectively arterIALIZes the blood [17, 18] and avoids the hazard of arterial cannulation which is ethically unacceptable for control subjects or patients with coagulopathy. Oxygen saturation (\( \text{SpO}_2 \)) of 95–97% was accepted as evidence of adequate arterialization. The femoral vein was cannulated antegrade under sterile conditions using an Abbocath-T 16G × 5.5 inch Teflon cannula with 2% (w/v) lignocaine as local anaesthetic. A constant infusion of stable-isotope-labelled \( l-[\text{1}^{13}\text{C}] \) leucine (KOR, Cambridge, MT, U.S.A.) was given at 1.0 mg h\(^{-1} \) kg\(^{-1} \). The primary bolus of leucine (1.0 mg/kg) and bicarbonate (0.16 mg/kg) to prime the bicarbonate pool [19] were added immediately before the infusion. The infusion and drip set were connected to the subject via an infusion pump (IVAC 170; IVAC Corp., San Diego, CA, U.S.A.) and the 3 h infusion commenced. Blood flow was measured by mercury-in-rubber strain gauge plethysmography [20, 21]. Limb and muscle volumes were measured as described by Jones & Pearson [22]. Measurements of skinfold thickness (biceps, triceps, subcapsular, suprailiac, thigh and calf) were made using Harpenden skin callipers to assess body fat [23, 24]. For measurement of \( ^{13}\text{C} \) enrichment of breath \( \text{CO}_2 \), samples were taken by using 20 ml evacuated tubes (Vacutainer; Becton-Dickinson, U.S.A.), connected to a 2 litre plastic bag inflated by the patient at intervals before and during the infusion. The rate of \( \text{CO}_2 \) production (\( \dot{\text{V}} \text{CO}_2 \)) was measured three times over the 3 h period by making a 10 min collection of expired gas into a 100 litre Douglas bag, and analysing a 2 litre portion of the gas for \( \text{CO}_2 \) by means of an infra-red absorbance analyser (Grubb Parsons Ltd, Newcastle-upon-Tyne, U.K.) and measuring the residual volume using a gasometer (Harvard Apparatus Ltd, Edenbridge, Kent, U.K.). The coefficient of variation for measurement of \( \dot{\text{V}} \text{CO}_2 \) using the Douglas bag was 5%.

**Protocol**

Before blood sampling for leg exchange, leg blood flow was measured and paired arterialized and venous samples were then drawn simultaneously for amino acid analysis.
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(glutamate, glutamine, alanine, leucine, isoleucine, valine, tyrosine, phenylalanine and 3-methylhistidine, L-[1-13C]-leucine and α-[l-13C]KIC) and blood gas analysis partial pressure of O₂ (partial pressure of CO₂, So₂). Blood and breath samples for 13CO₂ analysis were taken and leg blood flow measurements were made at the start of the period of investigation before infusion of 13C tracers (time 0), and at 120, 135, 150, 165 and 180 min thereafter. Plateau enrichments of 13CO₂ and α-[l-13C]KIC were obtained by 120 min. VCO₂ was measured three times during the 3 h sampling period; care was taken to acclimatize the patient to the Douglas bag on several occasions before taking measurements. Blood was added to lithium heparin tubes and kept on ice before centrifugation at 3000 rev/min for 20 min at 2°C. Thereafter the plasma was stored at −20°C before analysis.

Biochemical analysis

Partial pressure of O₂, haemoglobin concentration and total bicarbonate were measured using a Corning 189 blood gas analyser and So₂ was calculated. Blood amino acids were analysed on the Biotronik LC 5000 automated amino acid analyser (Biotech Instruments, Luton, U.K.) using strongly acid ion-exchange chromatography with elution and separation of amino acids by lithium citrate buffers. Amino acids were detected fluorometrically after post-column derivation with o-phthalaldehyde (e.g. fluxes of amino acids were calculated by multiplying arterialized and venous plasma concentrations of amino acids by plasma flow). Enrichment of plasma α-[l-13C]KIC was determined by selected-ion-monitoring gas chromatography–mass spectrometry (GCMS 10/20B Finnigan MAT, Hemel Hempstead, Herts, U.K.). Derivitization of keto acid samples for gas chromatography–mass spectrometry was performed by the method of Rocchiccioli et al. [25] as modified by Ford et al. [26]. α-KIC was analysed as its quinoxalinoltrimethylsilyl derivative (with ketovaleric acid as an internal standard) using electron-impact ionization. Enrichment of expired CO₂ with 13C was determined in breath using cryogenic distillation by isotope ratio mass spectrometry [27] using the Delta D instrument (Finnigan MAT, Hemel Hempstead, Herts, U.K.).

Statistics

Statistical analysis was performed by using the Mann–Whitney U-test.

RESULTS

Weight, leg volume, muscle volume, skinfold thickness values and percentage body fat were significantly reduced in patients with liver disease, reflecting their wasted state (Table 1). Mean arterial glutamate, tyrosine, phenylalanine and 3-methylhistidine concentrations (Table 2) were significantly raised (by 147%, 90%, 42% and 55%, respectively) and glutamine, alanine and branched-chain amino acids (BCAA) (Table 2) were significantly lower
(by 35%, 29% and 28%, respectively) in cirrhotic patients. Leg effluxes (i.e. arteriovenous difference multiplied by plasma flow, Table 3 and Fig. 1) of glutamine, alanine, valine, isoleucine and leucine were reduced (by 45%, 98%, 69% 50% and 50%, respectively) in cirrhotic patients. Efflux of tyrosine was increased by 45% but efflux of 3-methylhistidine was unchanged from that seen in normal control subjects. Since the blood flow was not significantly different between the cirrhotic patients and the normal subjects (Table 1), the changes in flux were mainly due to alterations in the arteriovenous differences between the two groups. Plasma insulin concentrations were elevated in cirrhotic patients relative to those in normal control subjects [18.2 (15.4-55.6) vs 9.8 (8.2-11.3) μ-units/ml, P< 0.05]. Plasma glucose concentrations were higher in cirrhotic than control subjects.

Whole-body protein turnover results are shown in Table 4. In cirrhotic patients whole-body leucine flux (i.e. in the fasted state, breakdown) appeared normal and whole-body leucine oxidation was elevated; therefore leucine available for whole-body protein synthesis must have been depressed (by 17%).

**DISCUSSION**

The amino acid profile seen in cirrhosis, of characteristically low arterial BCAA and elevated aromatic amino acid concentrations, is in keeping with previous reports [28, 29]. Aromatic amino acids such as tyrosine and phenylalanine are chiefly catabolized in the liver, and their metabolism is impaired in cirrhosis probably as a result of decreased functional liver mass or reduced enzyme activity. Leg efflux and plasma concentrations of BCAA were both low in cirrhotic patients. Previous workers have reported normal values for whole-body leucine kinetics in patients with cirrhosis [30-32] but we did not find this. The methodology used was similar in all

Table 1. Arterial amino acid concentrations

<table>
<thead>
<tr>
<th>Amino acid concn. ([μmol/])</th>
<th>Normal control subjects (n = 11)</th>
<th>Cirrhotic patients (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>40.8 (16.6-105.0)</td>
<td>148.9* (26.1-245.5)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>513.1 (492.8-700.4)</td>
<td>420.9* (219.0-625.9)</td>
</tr>
<tr>
<td>Alanine</td>
<td>167.0 (157.3-324.2)</td>
<td>117.6* (112.0-227.7)</td>
</tr>
<tr>
<td>Valine</td>
<td>358.4 (130.4-243.5)</td>
<td>31.5* (78.1-179.7)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>144.8 (35.8-55.2)</td>
<td>92.7* (18.4-78.7)</td>
</tr>
<tr>
<td>Leucine</td>
<td>113.6 (113.6-220.8)</td>
<td>79.8* (65.7-127.4)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>39.2 (28.4-55.5)</td>
<td>3.4 (64.2-104.6)</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>2.12 (2.12-5.90)</td>
<td>66.4* (4.5-9.3)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>48.8 (41.7-54.3)</td>
<td>66.4* (44.5-75.4)</td>
</tr>
</tbody>
</table>

Table 3. Efflux of amino acids

Values are shown as medians with the range in parentheses. A minus sign denotes efflux. Statistical significance: *P<0.05, **P<0.01 compared with normal control subjects.

<table>
<thead>
<tr>
<th>Amino acid efflux (nmol min⁻¹ 100 g⁻¹ of leg tissue)</th>
<th>Normal control subjects (n = 11)</th>
<th>Cirrhotic patients (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>38 (12 to 47)</td>
<td>18.5 (0.2 to 78.4)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>243.1 (42.3 to 322.1)</td>
<td>66.4** (23.9 to 351.2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>- 53.4 (-10.8 to -167.5)</td>
<td>- 4.6* (-92 to -39.8)</td>
</tr>
<tr>
<td>Valine</td>
<td>- 11.5 (-0.9 to -7.2)</td>
<td>- 2.7* (24 to -19.5)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>- 11.8 (-0.5 to -27.7)</td>
<td>- 11.6 (-3.5 to -11.6)</td>
</tr>
<tr>
<td>Leucine</td>
<td>- 11.8 (-1.8 to -53.8)</td>
<td>- 6.5* (-0.6 to -19.1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>- 4.2 (-2.2 to -7.7)</td>
<td>- 9.2* (-1.4 to -19.1)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>- 5.6 (-1.4 to 12.9)</td>
<td>- 28.4 (-0.4 to -28.4)</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>- 0.20 (-0.10 to -0.28)</td>
<td>- 0.06 (-2.7 to -1.59)</td>
</tr>
</tbody>
</table>

Fig. 1. Exchange of amino acids across leg tissue in normal control subjects (○) and cirrhotic patients (●). Results are medians. Statistical significance: *P<0.05, **P<0.005 compared with normal control subjects. Abbreviation: 3-MeHis, 3-methylhistidine.
studies except that our calculations of whole-body oxidation and flux were based on calculations for α-KIC and not leucine. Our values for leucine oxidation in control subjects do not differ significantly from those of Shanbhogue et al. [30], but are higher than those obtained by Mullen et al. [31], who used a much lower priming dose and infusion rate of [l-13C]leucine. We suggest that one reason for the differences seen in our results is that our control subjects do not differ significantly from those of previous reports in which patients were probably of normal weight [31]; in one report no patient details are supplied [32]. Shanbhogue et al. [30] studied patients with end-stage liver disease undergoing liver transplantation, but only four out of 32 patients had alcoholic cirrhosis, 94% had moderate or severe ascites and 22% encephalopathy at the time of study, thus providing a different study population. Furthermore, the control population in that study was not composed of ‘normal’ subjects and were undergoing abdominal surgery for unspecified reasons.

The markedly reduced efflux of alanine and glutamine from leg tissue coupled with depressed leg efflux of BCAA suggests that oxidation of BCAA derived from muscle protein breakdown was low, since alanine and glutamine are mainly synthesized in muscle, and since muscle is normally the primary site of BCAA catabolism [33], mostly endogenous to the muscle. However, whole-body leucine oxidation was increased, suggesting that there were increased BCAA in sites other than skeletal muscle. The alteration of the BCAA profile in cirrhotic patients may also be partly explained by an alteration in hormone balance. Increased plasma insulin and glucagon concentrations have been reported in cirrhotic patients [4, 34] due to hypersecretion resulting from reduced hepatic sensitivity rather than from decreased hormone degradation [34]. Increased insulin concentrations have been shown to inhibit BCAA decarboxylation in muscle but to enhance BCAA decarboxylation in fat [35], but such an effect can only be part of the explanation for the present results if there was a specific acceleration of leucine catabolism in adipose tissue, since adipose tissue was reduced.

Leg tyrosine efflux was increased by 45% in cirrhosis, suggesting that a state of net negative protein balance existed in the leg, which is 70–80% skeletal muscle by weight. Leg 3-methylhistidine efflux was not elevated above normal, indicating that muscle myofibrillar break-

down was not increased. The data therefore suggest that the predominant mechanism of muscle wasting in the legs of cirrhotic patients is a fall in protein synthesis. Since muscle contributes substantially to whole-body protein turnover, this is consistent with our whole-body results which provide independent evidence of a depression in protein synthesis, but not breakdown, in cirrhosis.

What is the mechanism for the reduced muscle protein synthetic rate in cirrhosis? Several possibilities exist: nutritional factors, hormone imbalance, alcoholic toxicity, neuropathy and immobility. Patients with cirrhosis (usually of alcoholic aetiology) often have clinical evidence of malnutrition [36]. A wide variety of vitamin and mineral deficiencies have been recognized in alcoholics, including those of thiamine, folic acid, pyridoxine, nicotinic acid, vitamin B₁₂, ascorbic acid, phosphate, calcium, magnesium and zinc [36]. Muscle fibre necrosis has also been described in association with type II fibre atrophy as a result of osteomalacia, which may complicate cirrhosis [37]. Protein-calorie malnutrition is recognized as causing a reduction in muscle protein synthesis and this may partly explain our findings, especially with patients on a low protein diet to control liver failure.

In cirrhosis an abnormal hormone milieu exists which may alter protein synthesis in muscle with both an increase in insulin and glucagon, and a reduction in the insulin/glucagon ratio [38]. Studies in animals, both in vitro [39, 40] and in vivo [41], provide evidence of a stimulatory effect of insulin on muscle protein synthesis, and an inhibitory effect on protein breakdown and, although some studies in adult man suggest that insulin may not have a simulatory effect [42] on protein synthesis, recent findings from our laboratory show that insulin is anabolic when sufficient amino acids are available (W. M. Bennett & M. J. Rennie, unpublished work). In cirrhosis, peripheral insulin resistance may also occur, at least with respect to carbohydrate metabolism. However, cirrhotic patients have been shown not to be resistant to insulin with respect to leucine metabolism [32]. Evidence for the effect of glucagon in vivo on protein synthesis and breakdown has been conflicting [38, 42, 43], although it is generally regarded as a catabolic hormone which is consistent with our results. Cortisol concentrations have been reported to be normal in cirrhosis [44] and may play little role in the effects seen here.

Although the predominant clinical form of nerve damage in alcoholics is a distal sensory neuropathy, there
is also substantial evidence for a motor component, as shown by slowed motor nerve conduction, reduced H reflex amplitudes, and reduced conduction velocities in the proximal segments of peripheral nerves [45, 46]. Both demyelination and axonal loss have been found in peripheral nerves of chronic alcoholics with muscle wasting [47]. The effect of motor denervation is to produce muscle atrophy of groups of muscle fibres which probably produces a fall in protein synthesis as observed in animal models. Alcoholic muscle wasting may also occur in the absence of peripheral neuropathy as a primary myopathy. Needle biopsy shows that atrophy of type II fibres mostly occurs in alcoholics, possibly as a direct toxic effect of ethanol on muscle fibres [48]; this could further reduce the muscle protein synthetic rate. A certain degree of immobility may also occur in these patients, especially if complicated by ascites or liver failure. Immobility can also result in muscle atrophy due to a reduction in muscle protein synthesis [49]; some of our patients were studied after a period of inpatient management when they would have been relatively immobile.

In conclusion, the main finding from this study is that the predominant mechanism for muscle wasting in cirrhosis is a depression in protein turnover associated with a greater reduction in muscle protein synthesis.

REFERENCES

Muscle protein turnover in cirrhosis


