Whole body protein kinetics measured with a non-invasive method in severely burned children

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Abstract

Persistent and extensive skeletal muscle catabolism is characteristic of severe burns. Whole body protein metabolism, an important component of this process, has not been measured in burned children during the long-term convalescent period. The aim of this study was to measure whole body protein turnover in burned children at discharge (95% healed) and in healthy controls by a non-invasive stable isotope method. Nine burned children (7 boys, 2 girls; 54 ± 14 (S.D.)% total body area burned; 13 ± 4 years; 45 ± 20 kg; 154 ± 14 cm) and 12 healthy children (8 boys, 4 girls; 12 ± 3 years; 54 ± 16 kg; 150 ± 22 cm) were studied. A single oral dose of $^{15}$N-alanine (16 mg/kg) was given, and thereafter urine was collected for 34 h. Whole body protein flux was calculated from labeling of urinary urea nitrogen. Then, protein synthesis was calculated as protein flux minus excretion, and protein breakdown as flux minus intake. At discharge, total protein turnover was $4.53 ± 0.65$ (S.E.) g kg body weight$^{-1}$ day$^{-1}$ in the burned children compared to $3.20 ± 0.22$ g kg$^{-1}$ day$^{-1}$ in controls ($P = 0.02$). Expressed relative to lean body mass (LBM), the rates were $6.12 ± 0.94$ vs. $4.60 ± 0.36$ g kg LBM$^{-1}$ day$^{-1}$ in burn vs. healthy ($P = 0.06$). Total protein synthesis was also elevated in burned vs. healthy children, and a tendency for elevated protein breakdown was observed. Conclusion: Total protein turnover is elevated in burned children at discharge compared to age-matched controls, possibly reflecting the continued stress response to severe burn. The oral $^{15}$N-alanine bolus method is a convenient, non-invasive, and no-risk method for measurement of total body protein turnover.

1. Introduction

A persistent and extensive skeletal muscle catabolism and weakness is characteristic of severe burns. Persons admitted with burns >40% of total body surface area (TBSA) have been shown to have 80–200% increases in metabolic rate leading to a nitrogen deficit of up to 30 g day$^{-1}$ [1]. This stressed state imposes greater demands for amino acids from muscle protein breakdown [2], as muscle protein serves as the main source to replace blood amino acids taken up by other tissues [3]. The loss of muscle mass is known to be detrimental to survival [4] and rehabilitation [5] from burn.

Current methods to study muscle metabolism involve muscle biopsies and blood sampling. Although muscle is
interest, information about the overall whole body protein metabolic rate is of importance as the increased amino acid need may be caused by processes such as accelerated synthesis of proteins involved in wound healing, synthesis of acute phase proteins in the liver, synthesis of proteins involved in immune function, and stimulation of hepatic gluconeogenesis [3,6]. It is not known whether an increased whole body protein metabolic rate is partly responsible for the hypermetabolic state that can last for more than 12 months after the initial trauma.

Much of the knowledge about whole body protein metabolism after trauma has been obtained from N-balance studies. Kien et al. [7] determined whole body protein synthesis and breakdown rates more precisely by administering 15N-glycine orally or intravenously every 3 h for 48 h in acutely burned children. The study was performed within 2 weeks of the initial burn and whole body protein synthesis and breakdown were higher in patients with >60% TBSA burned compared to patients with <25% TBSA burned or to control children undergoing reconstructive surgery. Positive correlations with %TBSA burn, % 3rd degree burn, and % open wound were also found. Seven patients repeated the study 3–6 weeks after the injury and showed a decline in protein synthesis and breakdown. Thereafter, three patients were studied again after about 88, 52 and 57 days postburn and had higher rates of breakdown again. This limited number of cases made analysis difficult, and whole body protein metabolism has not been previously measured in burned children during long-term convalescence. During the 30 years since this study was performed great improvements have been made in the care for burn patients [8,9]. Thus, the recovery in protein metabolism after burn may have also improved.

Recently, a non-invasive method with pulse ingestion of 15N amino acids has been used to measure whole body protein turnover in healthy [10,11] and obese children [12,13]. This is of advantage, as the children can be free living during the study. This oral dose method has also been used in critically ill children [14].

Thus, the aim of this study was to measure whole body protein turnover in burned children at discharge, defined as 95% healed, and in healthy control children by a non-invasive stable isotope method using 15N-alanine ingestion. We hypothesized that there would be an elevated protein turnover in burned children at discharge compared to healthy age-matched children.

2. Subjects and methods

2.1. Study design

Nine burned children (7 boys, 2 girls); and 12 healthy unburned children (8 boys, 4 girls) participated in the study. Table 1 shows the demographics for the two groups which were matched for age. The burned children were recovering from a 54 ± 14 (S.D.)% TBSA burn (37 ± 23% TBSA 3rd degree burn). Each child took part in a stable isotope study which included ingestion of a labeled amino acid, followed by 34 h of urine collection. In addition each child underwent a dual-energy X-ray absorptiometry (DEXA) scan. Subjects and families were thoroughly instructed about the procedures before written assent (child) and/or consent (parent/guardian) were obtained. A parent or guardian was included in the performance of all parts of the study. The protocol was approved by the Institutional Review Board at UTMB.

2.2. Isotope study

Fig. 1 shows the timeline of the isotope study. Subjects and families were carefully instructed by a dietician about appropriate procedures for recording diet intake and collecting urine. Food models were used when instructing on estimation of food portion sizes. Generally, studies were done in a weekend, starting Friday evening and lasting for 34 h.

The evening meal was recorded by the parent/guardian and subject and later recalled together with a dietician. Thereafter no food was consumed until in the morning the next day (Fig. 1). At least 2 h after the evening meal, a spot urine sample was collected for analysis of background urea enrichment. Thereafter subjects ingested a test drink containing 16 mg/kg body weight of 15N-alanine, i.e., 2.7 mg of 15N/kg (Cambridge Isotope Laboratories, Andover, MA), dissolved in water. A non-caloric flavor was added to the drink. During the next 10 h, the subject collected all urine in a container, including the morning urine the following day. No food or drink was allowed during this period, except for water intake. For the next 24 h, the subject continued collecting all urine in another

<table>
<thead>
<tr>
<th>Table 1 – Physical characteristics of all subjects.</th>
<th>Burns (n = 9)</th>
<th>Healthy controls (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>7:2</td>
<td>8:4</td>
</tr>
<tr>
<td>Age</td>
<td>12.9 ± 4.0</td>
<td>12.3 ± 2.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>45.0 ± 20.0</td>
<td>54.4 ± 15.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>153.7 ± 14.1</td>
<td>150.3 ± 22.2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19.0 ± 3.9</td>
<td>22.8 ± 4.7*</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>33.0 ± 12.4</td>
<td>38.7 ± 12.8</td>
</tr>
<tr>
<td>Lean body mass index (kg/m²)</td>
<td>14.1 ± 1.9</td>
<td>16.1 ± 3.6</td>
</tr>
<tr>
<td>Total body surface area burned (%)</td>
<td>54.2 ± 14.2</td>
<td>NA</td>
</tr>
<tr>
<td>Total body surface area 3rd degree burned (%)</td>
<td>37.3 ± 22.9</td>
<td>NA</td>
</tr>
<tr>
<td>Days post-injury</td>
<td>83 ± 46</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are mean ± S.D.

* P < .05 vs. burns.
container (Fig. 1). During this period, food and drinks were ingested as normal, recorded on forms by the parent/guardian and subject and later recalled together with a dietician. During the collection periods, urine containers were kept on ice or in a refrigerator. After the end of collection, total urine volume in each container was recorded. An aliquot of the urine was acidified with 6N HCl and later analyzed for urea nitrogen by a Dimension clinical chemistry system (Dade Behring Inc, Newark, DE). Aliquots were also frozen at –20 °C for later determination of total nitrogen content and urea enrichment.

2.3. Dual-energy X-ray absorptiometry (DEXA)

The children also underwent a full-body DEXA to determine body composition. All DEXA scans were performed on a Hologic QDR 4500 A system (Hologic, Inc., Bedford, MA).

2.4. Analyses

Urine samples for determination of urea enrichment were passed over a cation exchange column (AG 50W-X8 Resin, 100–200 mesh, H+ form; Bio-Rad Laboratories, Hercules, CA USA), eluted with 2N NH4OH and dried under vacuum with a Speed Vac (Savant Instruments, Farmingdale, NY, USA). Enrichment of urine urea was then determined on the tertiary-butyl dimethylsilyl (t-BDMS) derivatives using gas chromatography mass spectrometry (GCMS) (Hewlett-Packard 5973, Palo Alto, CA), and selected ion monitoring. Enrichments were expressed as tracer to tracee ratio corrected for background enrichment. Appropriate corrections were made for overlapping spectra [15]. Nitrogen (N) content in the urine was measured in duplicate using a micro-Kjeldahl technique (Tecator Kjeltec System, Hoganas, Sweden).

Food intake data was analyzed and protein intake computed by Nutritionist Pro™ Diet Analysis software (Axxya Systems, Stafford, TX).

2.5. Calculations

Fig. 2 shows a schematic of the model for calculation of whole body protein metabolism. The theoretical background is that nitrogen (N) is bound in body proteins, but there is also an active metabolic pool of nitrogen containing compounds [15]. The model assumes that these pools stay constant. The turnover or flux in the active metabolic pool of nitrogen is the rate that N goes in and out of this pool, i.e. flux is similar to the rate of which N goes into protein synthesis plus excretion, and also similar to the rate of which N comes into the pool from protein breakdown plus protein intake. By giving a labeled amino acid, the N in the active metabolic pool will be labeled, and either excreted or bound to protein. By measuring enrichment of the label in the end-product urea, total flux (excretion + incorporation into protein) can be calculated. By measuring total excretion of N, protein synthesis can also be calculated as flux minus excretion (see below), and protein breakdown as flux minus intake.

In more details, total N content in the urine was calculated as: Excretion = N-content in sample/sample volume × total urine volume per 34 h. Area under the curve (AUC) of 15N-urea enrichment was calculated as: 15N-urea AUC = Average enrichment in 0–10 h sample × 10 h + average enrichment in 10–34 h sample × 24 h. Enrichment of 15N AUC was then calculated as: 15N AUC = 15N-urea AUC/2, since there are two N-atoms in the urea molecule. Nitrogen flux, protein synthesis (PS), protein breakdown (PB) and protein net balance (NB) were thereafter calculated using the following formulas:

\[
\text{Flux (g N/day)} = \frac{15N-\text{alanine bolus (g) \times 15/90} \times 15N \text{AUC} \times 24 (h/day)},
\]

where the factor 15/90 is the % molecular weight of 15N in a 15N-alamine molecule.

\[
\text{Flux (g protein/day)} = \text{Flux (g N/day)} \times 6.25 \text{ g protein/g N}
\]
PS = Flux – protein excretion
PB = Flux – protein intake
NB = PS – PB

2.6. Statistical methods
Comparisons of parameters between groups were done by one-sided unpaired t-tests. Results were considered significant if P < 0.05. The results are presented as means ± S.E. unless otherwise noted.

3. Results
The burned children had a slightly lower body mass index (BMI) than their healthy controls, but no differences were found in lean body mass (LBM; Table 1). Total energy intake was 2038 ± 218 kcal day⁻¹ (50.0 ± 5.9 kcal kg⁻¹ day⁻¹) in the children with burns vs. 2082 ± 218 kcal day⁻¹ (40.8 ± 6.7 kcal kg⁻¹ day⁻¹) in the control group (NS vs. burn). Fat intake was 1.73 ± 0.27 and 1.62 ± 0.30 g kg⁻¹ day⁻¹ in burn vs. control (NS). Protein intake was sufficient according to recommendations in both the burned (2.07 ± 0.37 g kg⁻¹ day⁻¹) and control children (1.58 ± 0.21; NS vs. burn).

At discharge, total protein turnover (flux) was significantly higher in the burned children (4.53 ± 0.65 g kg body weight⁻¹ day⁻¹) compared to healthy controls (3.20 ± 0.22 g kg⁻¹ day⁻¹; P = 0.02 vs. burn; Fig. 3).

N-excretion was 1.52 ± 0.16 g kg⁻¹ day⁻¹ (2.01 ± 0.20 g kg LBM⁻¹ day⁻¹) in the burned children at discharge. This was not different from the healthy controls (1.39 ± 0.10 g kg⁻¹ day⁻¹; 1.96 ± 0.12 g kg LBM⁻¹ day⁻¹).

Total protein synthesis was higher in the burned children (3.01 ± 0.72 g kg⁻¹ day⁻¹) vs. healthy controls (1.81 ± 0.19 g kg⁻¹ day⁻¹; P = 0.04 vs. burn; Fig. 4, upper panel). There was also a tendency for elevated whole body protein breakdown (Burn: 2.46 ± 0.70 g kg⁻¹ day⁻¹; Controls: 1.62 ± 0.29 g kg⁻¹ day⁻¹; P = 0.12; Fig. 4, lower panel). We could not detect any correlation between protein intake and protein synthesis/protein turnover.

When protein flux, synthesis and breakdown rates were expressed relative to LBM, significance levels of differences between groups were weaker. Protein flux was 6.12 ± 0.94 vs. 4.60 ± 0.36 g kg LBM⁻¹ day⁻¹ in burn vs. healthy (P = 0.06; Fig. 5, upper panel). Protein synthesis rate was 4.11 ± 0.55 vs. 3.38 ± 0.30 g kg LBM⁻¹ day⁻¹ in burn vs. healthy (P = 0.07; Fig. 5, middle panel); and protein breakdown rates were 2.64 ± 0.30 g kg LBM⁻¹ day⁻¹ in burn vs. healthy (P = 0.15; Fig. 5, lower panel).

No correlations between %TBSA burned and protein turnover, synthesis or breakdown were found, while there was a tendency to correlations between %TBSA 3rd degree burn and protein turnover (r = 0.644, P = 0.061) and protein synthesis (r = 0.635, P = 0.066).

Protein net balance between synthesis and breakdown was 0.55 ± 0.30 g kg⁻¹ day⁻¹ in burned and 0.19 ± 0.25 g kg⁻¹ day⁻¹ in controls (P = 0.18). Protein net balance relative to LBM was 0.73 ± 0.38 vs. 0.29 ± 0.35 g kg LBM⁻¹ day⁻¹ in burn vs. healthy (P = 0.20).

Nitrogen balance measured as the difference between nitrogen intake and excretion was 0.089 ± 0.047 g kg⁻¹ day⁻¹ in burned and 0.028 ± 0.040 g kg⁻¹ day⁻¹ in controls (P = 0.17).
4. Discussion

The results of this study show that total protein turnover is elevated in burned children at discharge compared to age-matched controls, possibly reflecting a continued stress response to the burn injury and/or an anabolic recovery process.

Kien et al. [7] measured protein synthesis and breakdown in the acute stage of a burn injury using a constant administration of $^{15}$N-glycine. They found protein synthesis and breakdown rates to be 5.2 and 3.9 g protein kg$^{-1}$ day$^{-1}$, respectively, in patients with 25–59% TBSA burn, whereas corresponding numbers in patients with ≥60% TBSA burn were 7.7 and 6.3 g protein kg$^{-1}$ day$^{-1}$, respectively. The somewhat higher values in their study may be explained by the assessment of protein turnover at an earlier stage after burn injury in their patients compared to ours. They also found a correlation between %TBSA burn and 3rd degree burn vs. protein synthesis and breakdown, respectively, whereas we only found a weak correlation between %3rd degree burn vs. protein turnover and synthesis. This is reasonable since the patients in our study were 95% healed. In critically ill children with meningococcal sepsis, protein synthesis and breakdown values were about 9 g kg$^{-1}$ day$^{-1}$ as measured using an oral dose $^{15}$N-glycine[14]. In healthy children, also using $^{15}$N-glycine, variable protein synthesis and breakdown rates are reported [10,11,14], and lower values have surprisingly been found after walking [10] or resistance [11] training programs.

Corresponding to our results, Kien et al. [7] also found whole body protein synthesis to be higher than protein breakdown in children with burns. As a result, net balance between synthesis and breakdown is positive, indicating that protein is being retained in the body. This is in contrast to findings in muscle where a protein loss has been observed after burn injury [2]. Muscle may however serve as a reservoir for blood amino acids which may be taken up in other tissue with an increased demand of amino acids after burn, e.g., for wound healing, synthesis of acute phase proteins in the liver, synthesis of proteins involved in immune function, and stimulation of hepatic gluconeogenesis [3,6].

Even though protein breakdown was not found to be different between groups, it tended to be elevated in the burn children vs. control (Fig. 4). This tendency for an increase in breakdown is consistent with prolonged stress/catabolic response in the burn children. The calculation of breakdown is based on flux minus protein intake. Whereas flux can be determined precisely through labeling of urea, it is more difficult to measure protein intake exactly. This may lead to inaccuracy in the estimation of protein breakdown. In our study, registered dieticians helped us acquire and interpret the diet data, and the patient's parent or guardian was involved to ensure more precise registration, as it may be difficult for a child to keep accurate records.

Estimated protein intake was found to be at recommended level in both groups [16,17]. In burns, the intake was in the lower range of recommendations [17], even though it was still about 0.5 g kg$^{-1}$ day$^{-1}$ higher than in the control subjects. Better nutritional support and other burn care during the hospital stay may also have contributed to the 'improved' results in protein turnover data compared to the findings of Kien et al. [7] about 30 years ago.

4.1. Methodological considerations

There are few studies of protein metabolism in children. One reason for this is that most stable isotope methods to determine whole body protein metabolism are invasive and thus difficult to use in children. The non-invasive oral stable isotope method was first proposed more than 50 years ago, when a single oral dose of $^{15}$N-glycine was given to healthy humans and urinary urea used as end product [18]. Later, the
method was modified to use ammonia as end-product instead of (or combined) with urea for calculation of flux [19].

Most often $^{15}$N-glycine has been used as the label in the end-product method of measuring whole body protein turnover [10–14,20–23], whereas we used $^{15}$N-alanine instead. The alanine tracer has also been used by others during bed rest in adults [24]. The widely use of glycine partly has historical reasons, one being the availability of glycine in a time when availability of stable isotope tracers was limited and costly. Our rationale for using alanine as a tracer was that in contrast to glycine, which only to a limited extent participates in transamination reactions, alanine is easily mixed with the free N-pool, and it is the key mode by which N is transferred to the liver from peripheral tissue (muscle, gut and kidney) [25,26]. As a result, using $^{15}$N-alanine as tracer most likely leads to a more homogenous pool of N, which is precursor for both protein synthesis and urea and ammonia enrichments. In agreement with this, similar N enrichments of urinary urea and ammonia have been found when using $^{15}$N-alanine [27]. By using urea, higher enrichments can also be measured using gas chromatography mass spectrometry, whereas for ammonia enrichment, isotope ratio mass spectrometry is generally used.

Since the urea pool is larger than the ammonia pool, a longer sampling period is needed when using urea as the end-product compared to using ammonia. In the case of a shorter urea study, a blood sample for determination of urea concentration is often drawn for determination of changes in the pool. In the present study, we collected urine for 34 h after the bolus dose, and therefore did not need to draw any blood sample.

At an earlier stage after burn injury, significant amounts of N could be expected to be lost through the wounds, and therefore it would be more important to also measure the integumental N losses at that stage [7]. Since we were studying children at the time of discharge, defined as 95% healed, this was of less importance, and we assumed this loss to be similar between groups. In most conditions, integumental and faecal loss of N can be neglected, since the losses are small in comparison with the flux.

Compared to using a constant infusion of stable isotope tracer, the oral bolus dose is a convenient and non-invasive method that can be applied under free living conditions. Further, the cost is relative low and larger groups can also be studied. Thus, important information can be gleaned about protein turnover in burns, as well as in other populations.

One would expect the differences in protein turnover, synthesis and breakdown between our groups to be larger when expressed relative to LBM compared to whole body mass, since the burned children have lower LBM, but greater flux. However, we observed the opposite, perhaps due to a somewhat larger variability in LBM vs. whole body mass measurements.

5. Conclusion

We conclude that total protein turnover is elevated in burned children at discharge compared to age-matched controls, reflecting the continued stress response to a severe burn and/or an anabolic recovery process. The oral $^{15}$N-alanine bolus method is a convenient, non-invasive, and no-risk method for measurement of total body protein turnover, and is a promising method to measure response to interventions, such as exercise or anabolic agents, on whole body protein kinetics.

Conflict of interest

None.

Acknowledgments

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