Prolonged activation of the branched-chain 
$\alpha$-keto acid dehydrogenase complex
in muscle of zymosan treated rats

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Abstract. Whole-body oxidation rates of branched chain amino acids (BCAA) are increased during catabolic diseases. A significant role for muscle in this feature has been suggested and, therefore, activities of the rate limiting enzyme in the degradative pathway of the BCAA in muscle were investigated in a catabolic rat model (intraperitoneal zymosan injection). Both actual and total activities of the branched chain $\alpha$-keto acid dehydrogenase complex (BC-complex) were measured in skeletal muscle of zymosan treated rats and compared with values measured in pair fed and ad libitum fed controls. The actual activity and the percentage of the enzyme in the active form were increased 2 and 6 days after the zymosan challenge. Total activity of the BC-complex and the activities of mitochondrial marker enzymes were reduced 2 days after zymosan treatment. We conclude that zymosan treatment leads to (1) a reduction of the mitochondrial content in skeletal muscle and (2) a prolonged activation of the BC-complex in muscle which may explain enhanced oxidation of BCAA during catabolic diseases.

Keywords. Branched-chain $\alpha$-keto acid dehydrogenase, critical illness, mitochondria, muscle wasting, zymosan.

Introduction

Increased oxidation rates of branched chain amino acids (BCAA) have been repeatedly reported [1–9] to be part of the metabolic response during catabolic diseases (e.g. sepsis, trauma and cancer). This increased oxidation together with the observation that BCAA stimulate protein synthesis and reduce protein breakdown in muscle in vitro [10], have led to many therapeutic trials in which BCAA enriched nutritional regimes were studied in patients with sepsis and trauma. Some of the early studies reported a positive effect on nitrogen balance in a small number of patients [11,12], but no positive effects have been observed in more recent, better controlled clinical trials [13].

Recently we described a catabolic rat model showing an acute phase of critical illness for 2 days followed by prolonged recovery after an intraperitoneal injection with zymosan [14]. The model is characterized by severe muscle wasting during the acute phase. During the following recovery phase no signs of critical illness are observed any more but several changes (e.g. decreased muscle protein mass, decreased concentrations of muscle glutamine and increased liver size) indicate that the rats are not fully recovered 6 days after the zymosan challenge. The model seemed suited for the study of biochemical mechanisms behind metabolic derangements both during an acute phase of catabolic shock and a prolonged recovery phase, as commonly observed in critically ill patients [14]. Also, concentrations of plasma and muscle branched chain amino acids are reduced in the recovery period.

The branched chain $\alpha$-keto acid dehydrogenase complex (BC-complex) is the rate limiting enzyme in the degradative pathway of BCAA in muscle. Here we investigated whether the activity of this enzyme is enhanced in muscle of zymosan treated rats, in an attempt to provide a mechanism for the increased oxidation of BCAA during critical illness. The activity of the BC-complex is regulated by a phosphorylation/dephosphorylation cycle with phosphorylation causing inactivation. Therefore, both actual and total activities of the BC-complex were determined. Measurements were made 16h, 2 days and 6 days after treatment to investigate both acute and long-term effects.

To investigate whether changes in total activity of the BC-complex were related to changes in mitochondrial content, activities of two other mitochondrial enzymes (cytochrome c oxidase and citrate synthase) were also measured. The activities of these latter enzymes represent maximal activities and can be used, therefore, and indicators of mitochondrial content in muscle.
Materials and methods

Male SPF Lewis rats of approximately 200 g were supplied by the central laboratory animal facilities of the University of Limburg. The rats were individually housed and kept in a controlled environment (12 h light cycle, 21-22°C and 50-60% humidity). Rats were fed a standard laboratory chow (SRM-A, Hope Farms, the Netherlands) containing (w/w) approximately 28% protein, 7% fat, 54% carbohydrates, 4% fibres and 7% minerals with a trace element and vitamin supplement. The rats were allowed to acclimatize for 1 week. The experiments were approved by the animal experiments committee of the University of Limburg.

Previously we have reported concentrations of amino acids and protein synthesis rates in zymosan treated rats [14]. The same rats were used for the measurements described in the present paper.

Rats were injected intraperitoneally with zymosan (50 mg per 100 g body weight) suspended in liquid paraffin (25 mg mL⁻¹). Zymosan administration reduced food intake and, therefore, the paraffin-injected control rats were pair-fed. Pair feeding was performed in three periods during 24 h (from 08.00 until 15.00 hours, 15.00 until 22.00 hours, and from 22.00 until 08.00 hours) to ensure that the pair-fed rats would not eat all the food at once and consequently would be starving for the remainder of the day. Also an ad libitum fed non-treated control group was included to study the effect of pair feeding. These rats were studied on day 3 to make them closely comparable to the other groups. All groups were matched for age and initial body weight. During the experiment food intake and body weight were determined daily.

On the day of the measurements pair feeding was performed at 07.00 hours and food was withheld in all groups from 08.00 until 12.00 hours. Sampling of muscle tissue and measurements of the BC-complex activities were done between 12.00 hours and 14.00 hours. Activities of the BC-complex, cytochrome c oxidase and citrate synthase were measured in muscle of zymosan treated, pair-fed, and ad libitum fed control rats 16 h, and 2 and 6 days after treatment.

Animals were killed by cervical dislocation and the gastrocnemius muscle was rapidly removed, weighed, and about 50 mg of the muscle sample was transferred to ice cold SET buffer (0.25 M sucrose, 2 mM EDTA and 10 mM Tris/HCl; pH = 7.4) for preparation of a homogenate to measure enzyme activities. Homogenates (5%) of the fresh muscle sample were made in ice cold SET buffer within 1 min after sampling, using a Teflon–glass Potter–Elvehjem homogenizer. Both the total activity and the actual activity of the BC-complex were immediately determined as described previously [15]. The enzyme complex is active in the dephosphorylated form. To measure the actual activity, both kinase and phosphatase of the BC-complex were inhibited. This was attained by incubation with 5 mM ADP (Kinase inhibition) and with 50 mM NaF (phosphatase inhibition). For measuring total activity, only the kinase was inhibited and, therefore, all the enzyme dephosphorylated. Activities in (nmol min⁻¹ g wet weight⁻¹) were calculated from the ¹⁴CO₂ production during incubation with [1-¹⁴C]-4-methyl-2-oxopentanooate (α-keto acid of leucine) for 10 min at 37°C. The same homogenate was used to determine activities of cytochrome c oxidase and citrate synthase. These enzymes were used as mitochondrial marker enzymes in order to obtain information regarding changes in mitochondrial content. Activities of cytochrome c oxidase were measured as described previously [16]. Citrate

![Figure 1](image1.png)

**Figure 1.** Food intake (g day⁻¹) of control and zymosan treated rats. The zymosan challenge was given on day 6 of the experiment. *Significantly different from control (P < 0.05). □, zymosan; A, control.

![Figure 2](image2.png)

**Figure 2.** Cumulative changes in body weight (g) of control, pair-fed and zymosan treated rats. Rats were injected with either zymosan suspended in paraffin or with paraffin only (pair-fed). Control rats were not injected. Changes were calculated from initial body weight and body weights attained every morning during the experiment. All values of the zymosan treated and the pair-fed rats are significantly different from those of the control rats, except on day 0 and 1 for the pair-fed rats. †Significant difference between zymosan treated and pair-fed rats (P < 0.05). □, zymosan; A, pair-fed; A, control.
synthase activities were measured as described by Shepherd & Garland [17] following four times freezing (liquid nitrogen) and thawing (water bath) and ultrasonication of the sample.

All measurements were done in duplicate except for the actual activities of the BC-complex which were done in triplicate. Values shown are mean (range). Statistical analysis of differences between zymosan treated and pair-fed animals and between the experimental groups and the ad libitum fed control rats were performed using the Mann–Whitney U-test. Significance was set at $P < 0.05$.

Results

Following the zymosan challenge rats almost stopped eating for 2 days after which the intake gradually increased to approximately 80% of normal (Fig. 1). Both the zymosan injected and pair-fed rats lost body weight for 3 days (Fig. 2). An increase in body weight was observed for the following 3 days, but no catch-up growth was present.

The actual activity of BC-complex in muscle of the zymosan treated rats was increased 16 h after treatment in comparison with the fed control rats and on day 2 and 6 in comparison with both the fed and pair-fed control rats (Table 1). The increased actual activity of the pair-fed group 16 h after treatment was back to normal on day 2 and marginally decreased on day 6 (Table 1). The total activity of the BC-complex (total amount of enzyme) was decreased in the zymosan treated animals 16 h and 2 days after treatment (Table 1). The percentage of the enzyme in the active form was increased in the zymosan treated rats 16 h after treatment in comparison with the fed control rats and on 2 and 6 days after treatment in comparison with both the pair-fed and fed control rats (Table 1).

The reduction in total activity of BC-complex on day 2 was paralleled by a decrease in the activities of both cytochrome c oxidase and citrate synthase (Table 2). Activities of cytochrome c oxidase and citrate synthase stayed low until 6 days after treatment. Activity of cytochrome c oxidase but not of citrate synthase was decreased in the pair-fed group in comparison with the fed control rats 16 h, 2 and 6 days after treatment (Table 2).

Discussion

Enhanced oxidation of BCAA has been reported to occur during severe catabolic diseases. In an animal model of sepsis in which rats were injected with live E. coli bacteria, a 1.4–1.5-fold increase in the oxidation rate of leucine has been observed [6,9]. Also in patients with sepsis and burn injury $^{12}$C-leucine oxidation was increased 2–2.6 fold [2]. Several studies have indicated that the increased oxida-

### Table 1. Total and actual activity (nmol min$^{-1}$ g wet weight$^{-1}$) and the percentage active form of the BC-complex in muscle on 16 h, 2 days and 6 days after treatment

<table>
<thead>
<tr>
<th>Activity of BC-complex</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual activity</td>
<td>0.6</td>
<td>0.9*†</td>
<td>1.4*</td>
<td>1.1*†</td>
<td>0.6</td>
<td>1.2*†</td>
<td>0.4*</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Total activity</td>
<td>31.2</td>
<td>19.9†</td>
<td>25.7</td>
<td>14.9†</td>
<td>24.8</td>
<td>26.7</td>
<td>26.7</td>
<td>20.7</td>
<td>20.7</td>
<td>26.7</td>
<td>20.7</td>
<td>20.7</td>
</tr>
<tr>
<td>Active form (%)</td>
<td>2.1</td>
<td>4.5*</td>
<td>5.7*</td>
<td>9.2*†</td>
<td>2.9</td>
<td>5.0*†</td>
<td>1.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Values are given as mean (range) of six–eight rats. * Significantly different from control; † significant difference between zymosan treated and pair-fed rats ($P < 0.05$).

### Table 2. Activities of the mitochondrial enzymes cytochrome c oxidase and citrate synthase (nmol min$^{-1}$ g wet weight$^{-1}$) in muscle on 16 h, 2 days and 6 days after treatment

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
<th>Control</th>
<th>Zymosan</th>
<th>Pair-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>172</td>
<td>205†</td>
<td>168*</td>
<td>50*†</td>
<td>128*</td>
<td>75*†</td>
<td>125*</td>
<td>125*</td>
<td>125*</td>
<td>125*</td>
<td>125*</td>
<td>125*</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>26</td>
<td>29</td>
<td>20</td>
<td>14*†</td>
<td>23</td>
<td>17*†</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

Values are given as mean (range) of six–eight rats. * Significantly different from control; † significant difference between zymosan treated and pair-fed rats ($P < 0.05$).
tion of BCAA involves skeletal muscle. Woolf et al. [8] measured arteriovenous differences of amino acids across the leg in a dog model with E. coli bacteremic shock. From an increased release of phenylalanine (indicating an increased protein breakdown rate) together with a decreased release of BCAA they concluded that BCAA are oxidized faster in muscle of animals with bacteremic shock. Similar results have been reported in septic patients [1]. Increased oxidation of BCAA has been found in incubated muscles of animal models with severe protein catabolism. Skeletal muscles and diaphragms incubated with $^{14}$C-leucine released more $^{14}$CO$_2$ when obtained from burn injured [5,7] and tumour bearing rats [3,4] than when taken from control rats.

The first enzyme in the oxidative pathway of BCAA is the BCAA aminotransferase. This enzyme catalyses a reversible transamination step to branched chain $\alpha$-keto acids and is present in abundance in muscle. The second enzyme in the degradative pathway of the BCAA, the BC-complex catalyses the irreversible decarboxylation of the branched-chain $\alpha$-keto acids to branched-chain acylCoA esters. The activity of the enzyme is regulated by a phosphorylation/dephosphorylation cycle with only 2–3% of the enzyme being active in skeletal muscle. The BC-complex, therefore, is the rate-limiting enzyme in the degradative pathway of BCAA in muscle and may play a role in the increased oxidation of BCAA observed during catabolic diseases. In the present study modest but prolonged activation of the BC-complex in muscle was observed in skeletal muscle of zymosan injected rats. This may provide a bio-

![Graph](image)

**Figure 3.** Sum of branched-chain amino acids (leucine, isoleucine and valine) concentrations in gastrocnemius muscle (nmol g$^{-1}$ wet weight) of control, pair-fed and zymosan treated rats. Values are given as mean (range of six–eight rats). # Significantly different from control. † Significant difference between zymosan treated and pair-fed rats. Data are adapted from [14] in which the same rats were used as in the present study. #, zymosan; †, pair-fed; ‡, control.

chemical mechanism for the increased oxidation of BCAA during catabolic diseases. Also, the decreased concentrations of BCAA in muscle (Fig. 3) and possibly plasma [14] in the zymosan treated rats 6 days after injection may be the result of this prolonged activation of the BC-complex in muscle.

Increased concentrations of BCAA were observed in rat muscle 16 h after zymosan treatment in comparison with pair fed and ad libitum fed rats (Fig. 3). This increase may lead to the observed activation of the BC-complex. A correlation between concentrations of BCAA and activity of the BC-complex has been observed before [18] and may be explained by inhibition of the kinase of the BC-complex by the branched chain $\alpha$-keto acids [19]. Net protein breakdown in muscle of the zymosan treated rats, indicated by a loss of muscle protein and decreased protein synthesis rates in the acute phase of illness [14] seems to be the cause for the increased concentrations of BCAA. However, an even greater activation of the BC-complex in muscle of the pair-fed rats on 16 h in combination with normal BCAA concentrations (Fig. 3) indicate that other mechanisms must be involved. This could include increased concentrations of free ADP in muscle, which inhibit the kinase of the BC-complex [19] or effects of cytokines or stress hormones. Both cytokines and glucocorticoids have been reported to activate the BC-complex when administered to rats [20,21]. Zymosan is a potent activator of monocytes and macrophages [22] and, therefore, enhanced production of cytokines is likely to occur in the zymosan treated rats. Von Asmuth et al. [23] have reported increased circulatory levels of TNF and interleukin-6 several hours following zymosan injection in mice.

Both cytochrome c oxidase and citrate synthase are mitochondrial marker enzymes and can be used to estimate changes in mitochondrial content of tissues. The decreased amount of BC-complex (total activity) 2 days after zymosan treatment may, therefore, be the result of a reduced mitochondrial content indicated by the reduced activities of cytochrome c oxidase and citrate synthase. Both protein and water content of gastrocnemius in the zymosan treated rats were not changed [14] indicating that the decreases in enzyme activities are not the result of water retention. Fatigue and muscle weakness are characteristic features observed during recovery from surgery and severe catabolic diseases [24]. The decreased activities of cytochrome c oxidase and citrate synthase in muscle of zymosan treated rats on days 2 and 4 indicate a possible role of impaired mitochondrial content in the reduced muscle function and occurrence of fatigue. Changes in mitochondrial content in skeletal muscle may be the result of changes in the fibre type composition (Type I/Type II ratio).

To summarize, we report low activities of mitochondrial marker enzymes and a prolonged activation of the BC-complex in skeletal muscle of zymosan treated rats. This activation of the BC-
complex may supply a mechanism for increased BCAA oxidation during catabolic diseases.

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References