

Protein metabolism in slow- and fast-twitch skeletal muscle during turpentine-induced inflammation

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Summary

The aim of our study was to evaluate the differences in protein and amino acid metabolism after subcutaneous turpentine administration in the soleus muscle (SOL), predominantly composed of red fibres, and the extensor digitorum longus muscle (EDL) composed of white fibres. Young rats (40–60 g) were injected subcutaneously with 0.2 ml of turpentine oil/100 g body weight (inflammation) or with the same volume of saline solution (control). Twenty-four hours later SOL and EDL were dissected and incubated in modified Krebs–Heinseleit buffer to estimate total and myofibrillar proteolysis, chymotrypsin-like activity of proteasome (CHTLA), leucine oxidation, protein synthesis and amino acid release into the medium. The data obtained demonstrate that in intact rats, all parameters measured except protein synthesis are significantly higher in SOL than in EDL. In turpentine treated animals, CHTLA increased and protein synthesis decreased significantly more in EDL. Release of leucine was inhibited significantly more in SOL. We conclude that turpentine-induced inflammation affects more CHTLA, protein synthesis and leucine release in EDL compared to SOL.

Keywords

amino acids, fast-twitch, inflammation, leucine, proteasome, protein synthesis, proteolysis, slow-twitch

There are two basic types of skeletal muscle fibres (I and II) which differ in both physical and biochemical properties due to alternate expression of myosin heavy chain (MHC) isoforms (Rivero *et al.* 1996). According to the dominantly expressed MHC isoform, they may be further divided into subtypes I, IC, IIC, IIA, IIAD, IID, IIDB and IIB. However, many fibres co-express different MHC isoforms in variable relative amounts, forming a continuum (Sant'ana Pereira *et al.* 1995). In general, type I fibres have small diameters (Rivero *et al.* 1998), they contain

higher levels of mitochondria and myoglobin, their contraction is more prolonged, their twitch rate is slower (Schiaffino & Reggiani 1996), they have more capillaries than type II fibres do and the main source of energy is blood glucose and free fatty acids. Type II fibres are thicker, they contain higher levels of myosin ATPase, their contraction is stronger and faster, muscle glycogen serves as the basic source of energy and the resistance to fatigue is not so high as in the case of type I fibres. With respect to these properties, type I fibres are also called red,

oxidative or slow-twitch fibres and type II fibres white, glycolytic or fast-twitch fibres.

Skeletal muscles have variable numbers of fast-twitch and slow-twitch fibres, the relative amounts are appropriately related to function, training and genetic set (Gollnick *et al.* 1972; Simoneau & Bouchard 1995; Kernell *et al.* 1998). The large back muscles, which are necessary for maintaining erect posture or breathing, or leg muscles, used for walking, such as the soleus muscle (SOL), are predominantly composed of slow-twitch fibres (Gupta *et al.* 1989), which allow them prolonged, steady contractions. In contrast, muscles which ensure delicate and rapid movements, e.g. muscles of the eye or muscles of the fingers, such as the extensor digitorum longus (EDL) muscles, are predominantly composed of fast-twitch fibres (Soukup *et al.* 2002).

Skeletal muscles are known to serve as the main source of amino acids in various pathological conditions, such as starvation, cancer, sepsis, or trauma providing amino acids, especially for acute-phase protein synthesis and gluconeogenesis. In severe illnesses, the enhanced amino acid release into the circulation is caused by increased proteolysis and decreased protein synthesis, resulting in muscle cachexia. A number of animal models provide evidence of the different responses of SOL and EDL to inflammation, but many of these have important limitations, particularly associated with the trigger for inflammation. Endotoxin administration is usually performed by constant infusion to prevent its rapid elimination from the body (Fish & Spitzer 1984). A high variability of response (Mela-Riker *et al.* 1988) is a disadvantage of bacteria application. Additionally, if bacteria are injected in bolus form, shock is often developed (Hau & Simmons 1977). Cecal ligation and puncture (CLP) is a complicated and invasive method requiring anaesthesia (Safranek *et al.* 2006).

It has been demonstrated that a simple and reproducible model, which causes localized inflammation (Laflamme & Rivest 1999), is subcutaneous (s.c.) turpentine administration. This agent has been used to study hormonal changes, hepatic regeneration and production of acute-phase mediators following acute inflammation (Kulkarni *et al.* 1985; Birch & Schreiber 1986; Woloski & Jamieson 1987; Okajima *et al.* 1997). To date however, little is known about effect of turpentine-induced inflammation on protein and amino acid metabolism in various types of skeletal muscle.

Considering the different fibre compositions and correspondingly different functions and metabolic features of SOL and EDL, different responses of SOL and EDL during turpentine-induced inflammation should be expected. The aim of our study was to evaluate the differences in protein and amino acid metabolism after s.c. turpentine admini-

stration in SOL (slow-twitch, red muscle) and EDL (fast-twitch, white muscle).

Methods

Animals

Male Wistar rats (body weight 40–60 g) obtained from Bio-Test (Konarovice, Czech Republic) were used in this study. The rats were housed under controlled conditions (12-h light-dark cycle, 22 °C, 55–65% relative humidity) with free access to standard laboratory chow and water. All procedures involving animals were performed according to guidelines set by the Institutional Animal Use and Care Committee of Charles University, Prague, Czech Republic.

Materials

L-[1-¹⁴C]Leucine was purchased from MP Biomedicals (Irvine, CA, USA); amino acids, cycloheximide, Folin-Ciocalteu's phenol reagent, turpentine oil, LLVY-MCA and albumin from Sigma Chemical (St Louis, MO, USA); hydroxide of hyamine from Packard Instruments (Meriden, CT, USA); Aminoplasmal 15 from B. Braun Medicals (Melsungen, Germany); MG-132 from Biomol (Hamburg, Germany). Other chemicals were purchased from Sigma Chemicals, Waters (Milford, MA, USA) and Lachema (Brno, Czech Republic).

Experimental design

The rats were injected by 0.2 ml of turpentine/100 g body weight in the dorsolumbar area. Control animals were injected with the same volume of 0.9% saline solution. After 24 h, the rats were anaesthetized with pentobarbital (6 mg/100 g body weight, intraperitoneally) and blood was withdrawn from the bifurcation of aorta. Administration of turpentine oil s.c. causes localized inflammation via necrosis (Wusteman *et al.* 1990). The dose of turpentine was determined on the basis of the study of Wusteman *et al.* (1990). The time interval was selected according to the experiments of Zarrabian *et al.* (1998) and Birch and Schreiber (1986) who reported the maximal levels of several acute phase proteins after 24 h following turpentine insult. SOL and EDL muscles of both legs were dissected according to Maizels *et al.* (1977). Isolated muscles were fixed to stainless steel clips to provide slight tension and immediately transferred to 2.5 ml of modified Krebs-Heinseleit bicarbonate buffer with 6 mM glucose and 2 mU/ml insulin (pH 7.4, 37 °C). Other components present in the medium were dependent on the measured parameter [protein synthesis and leucine

oxidation or proteolysis and chymotrypsin-like activity (CHTLA) of proteasome]. The medium was saturated with O₂/CO₂ (19:1). Muscles were preincubated for 30 min in a thermostatically controlled bath (37 °C) with a shaking device (70 cycles/min). After preincubation, muscles were quickly rinsed in 0.9% NaCl, blotted and transferred to a second set of vials containing fresh incubation media identical in composition and volume with the preincubation one. The viability of incubated muscles was previously confirmed in our laboratory (Safranek *et al.* 2003) as well as by other authors (Fang *et al.* 2005). Muscles from the right leg were always used for proteolysis and CHTLA determination, muscles from the left leg for protein synthesis and leucine oxidation assay.

Protein synthesis and leucine oxidation

Protein synthesis and leucine oxidation was measured after 1-h incubation of the muscle in 2.5 ml of medium enriched with amino acids in approximately physiological concentrations (Aminoplasmal 15 with added glutamine and tyrosine, total concentration 2.8 mM) and L-[1-¹⁴C]leucine (0.6 µCi/ml). At the end of the incubation period, 0.4 ml of hydroxide of hyamine was added to the well hanging above the incubation medium, the reaction was stopped by the addition of 35% (v/v) perchloric acid solution (0.2 ml) into the incubation medium and the flasks were shaken for 1 h to ensure complete absorption of ¹⁴CO₂ into the hyamine hydroxide. Muscles were removed from the incubation flasks, quickly rinsed in cold 6% (v/v) HClO₄, blotted, and homogenized in 0.6 ml of 6% (v/v) HClO₄. The homogenate was centrifuged for 5 min at 12,000 g and the pellet was used for other measurements. L-[1-¹⁴C]Leucine incorporation into precipitated proteins was estimated after their hydrolysis in 1 M NaOH. Protein synthesis and leucine oxidation was calculated using leucine specific activity in the incubation medium and expressed as nmol of incorporated leucine/g protein/h and nmol of oxidized leucine/g wet weight/h respectively. The radioactivity of the samples was measured with the liquid scintillation radioactivity counter LS 6000 (Beckman Instruments, CA, USA). The protein content of the samples was estimated according to Lowry *et al.* (1951).

Total and myofibrillar proteolysis

Total and myofibrillar protein breakdown was estimated after 2-h incubation of muscle in medium enriched with cycloheximide (0.5 mM), which prevented the reincorporation of released amino acids into proteins. As tyrosine is not

metabolized in the muscle, the amount released into the medium reflects total proteolysis in the muscle. The amount of 3-methylhistidine (3MH), a characteristic product of myofibrillar breakdown, released into the medium served for myofibrillar proteolysis calculation. The rates of amino acid release were estimated on the basis of their appropriate concentrations in the medium and weight of muscle. 3MH was quantified using a high performance liquid chromatography (HPLC) method (Waters) based on reaction with fluorecamine (Wassner *et al.* 1980; Lowell *et al.* 1986); other amino acid concentrations in the medium were determined by the HPLC method after precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen & Michaud 1993; Reverter *et al.* 1997).

Chymotrypsin-like activity of proteasome

The CHTLA of proteasome was determined using fluorogenic substrate SucLLVY-MCA (0.1 mM) according to the method of Gomes-Marcondes and Tisdale (2002) with the following modifications. Muscles were homogenized after incubation and the homogenate was centrifuged for 10 min at 18,000 g at 4 °C. Cellular supernatant (0.1 ml) was incubated with substrate (0.05 mM) in a total volume of 0.2 ml, with and without inhibitor, MG-132 (0.02 mM), for 1 h on ice. Fluorescence was determined with an excitation wavelength of 340 nm and an emission wavelength of 440 nm (Perkin Elmer luminescence spectrometer LS 50B). The standard curve was established for 7-amino-4-methylcoumarin (AMC) which permitted the expression of CHTLA activity as nmol AMC/g protein/h. The activity was adjusted for the protein concentration of the supernatant estimated according to Lowry *et al.* (1951). The differences after subtraction of inhibited from non-inhibited activities were used for the calculations.

Statistical analysis

Results are expressed as the mean ± SEM. *F*-test and two-sample *t*-test or ANOVA followed by Tukey–Kramer test (statistical software NCSS 2001; NCSS Raysville, UT, USA) were used for analysis of the data. Differences were considered significant at *P* < 0.05.

Results

Protein synthesis and leucine oxidation

Leucine oxidation was significantly higher in SOL of both intact and turpentine treated rats compared to EDL. The

Table 1 Basic parameters of protein and amino acid metabolism in EDL and SOL

	Control		Turpentine	
	EDL _c (n = 7)	SOL _c (n = 7)	EDL _t (n = 7)	SOL _t (n = 7)
Total proteolysis (nmol TYR/g wet wt/h)	109 ± 6	171 ± 7*	145 ± 5	219 ± 16 ^{†‡}
Myofibrillar proteolysis (nmol 3MH/g wet wt/h)	0.542 ± 0.033	1.167 ± 0.086*	0.845 ± 0.119	1.447 ± 0.157 [†]
CHTLA of proteasome (nmol AMC/g of protein/h)	1034 ± 71	2125 ± 125*	1640 ± 106	2436 ± 174 [†]
Leucine oxidation (nmol LEU/g wet wt/h)	45 ± 3	86 ± 7*	59 ± 9	116 ± 12 [†]
Protein synthesis (nmol LEU/g of protein/h)	2870 ± 136	3269 ± 166	1982 ± 77	2804 ± 271 [†]

SOL, the soleus muscle; EDL, the extensor digitorum longus muscle; TYR, tyrosine; 3MH, 3-methylhistidine; CHTLA, chymotrypsin-like activity; AMC, 7-amino-4-methylcoumarin; LEU, leucine.

Values are expressed as mean ± SEM.

ANOVA followed by Tukey–Kramer test.

**P* < 0.05 SOL_c vs. EDL_c.

[†]*P* < 0.05 SOL_t vs. EDL_t.

[‡]*P* < 0.05 SOL_t vs. SOL_c.

difference in protein synthesis between SOL and EDL of intact animals was not significant while in SOL of turpentine treated animals, this parameter was significantly higher. There were no statistical differences between control and turpentine treated rats neither in SOL nor in EDL (Table 1). The relative decrease in protein synthesis in turpentine group against the control group was significantly higher in EDL than in SOL while leucine oxidation changes were not statistically significant (Figure 1).

Total and myofibrillar proteolysis

Total and myofibrillar proteolysis was significantly higher in SOL of both intact and turpentine treated rats compared to EDL. Application of turpentine oil caused significant

increase in total proteolysis in SOL only. Myofibrillar proteolysis in the septic group remained unchanged against control in both types of muscle (Table 1). Relative changes in total and myofibrillar proteolysis in the turpentine group against the control were not statistically significant in both SOL and EDL (Figure 1).

Chymotrypsin-like activity of proteasome

The CHTLA of proteasome was statistically higher in SOL of both intact and turpentine treated rats compared to EDL. We detected no significant changes between the control and septic groups (Table 1). However, the relative increase in CHTLA in EDL of septic rats against control was statistically higher than in SOL (Figure 1).

Amino acid release into the incubation medium

Table 2 represents rates of amino acid release from SOL and EDL of turpentine treated and control animals. Most amino acids shared a significantly greater release into the medium from SOL than from EDL in both experimental groups. In SOL of turpentine treated animals, we observed a significant decrease in leucine release compared to the control while EDL was not affected. Figure 2 demonstrates relative changes in amino acid release from EDL and SOL of turpentine treated rats against the control. In EDL, release of asparagine, leucine and BCAA (branched chain amino acid – valine, leucine, isoleucine) increased while decreasing in SOL, resulting in a significant difference. In contrast, release of glycine decreased in EDL and increased in SOL. In SOL, release of alanine decreased while release of histidine and lysine increased significantly more than in EDL.

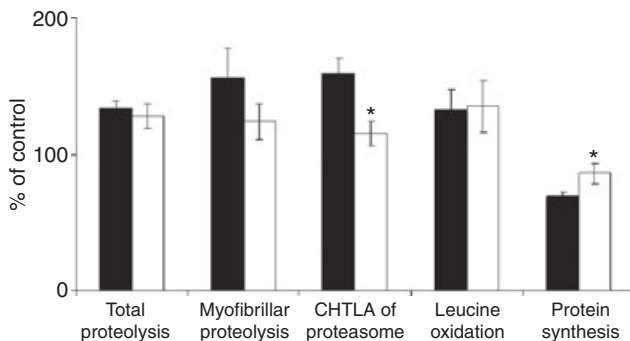


Figure 1 Relative effect of inflammation on basic parameters of protein and amino acid metabolism in EDL (filled bars) and SOL (open bars). Means of appropriate control values were used as 100%. Values are means ± SEM, *n* = 7 in each group. *F*-test followed by two-sample *t*-test; **P* < 0.05 vs. EDL. SOL, the soleus muscle; EDL, the extensor digitorum longus muscle; CHTLA, chymotrypsin-like activity.

Table 2 Release of amino acids into the medium (nmol/g wet wt/h)

	Control		Turpentine	
	EDL _c (n = 7)	SOL _c (n = 7)	EDL _t (n = 7)	SOL _t (n = 7)
ALA	739 ± 117	856 ± 108	595 ± 22	471 ± 25
ASN	105 ± 19	196 ± 27 [‡]	150 ± 11	135 ± 13
GLU	130 ± 23	330 ± 37 [‡]	143 ± 18	370 ± 36 [†]
GLN	1038 ± 139	1541 ± 218 [*]	1517 ± 72	2116 ± 176 [†]
GLY	807 ± 197	785 ± 102	712 ± 57	1063 ± 123 [†]
HIS	143 ± 13	202 ± 21	170 ± 7	282 ± 29 [†]
ILE	132 ± 20	208 ± 17 [‡]	156 ± 6	266 ± 21 [†]
LEU	261 ± 32	413 ± 32 [‡]	311 ± 11	232 ± 18 ^{†‡}
LYS	360 ± 40	417 ± 30	404 ± 29	584 ± 39 [†]
MET	73 ± 8	125 ± 10 [‡]	83 ± 12	146 ± 8 [†]
PHE	142 ± 19	222 ± 17 [‡]	167 ± 7	264 ± 20 [†]
PRO	282 ± 21	359 ± 14 [‡]	319 ± 14	457 ± 42 [†]
SER	376 ± 51	570 ± 39 [‡]	352 ± 76	740 ± 108 [†]
VAL	217 ± 30	332 ± 23 [‡]	245 ± 11	401 ± 33 [†]
BCAA	610 ± 82	953 ± 72 [‡]	712 ± 28	899 ± 72 [†]
TOTAL	4805 ± 729	6556 ± 695 [*]	5324 ± 353	7527 ± 691 [†]

SOL, the soleus muscle; EDL, the extensor digitorum longus muscle; BCAA, branched chain amino acids (valine, leucine, isoleucine); TOTAL, sum of all measured amino acids; ALA, ASN, GLU, GLN, GLY, HIS, ILE, LEU, LYS, MET, PHE, PRO, SER, VAL – standard amino acid abbreviations.

Values are expressed as mean ± SEM.

ANOVA followed by Tukey–Kramer test.

^{*}P < 0.05 SOL_c vs. EDL_c.

[†]P < 0.05 SOL_t vs. EDL_t.

[‡]P < 0.05 SOL_t vs. SOL_c.

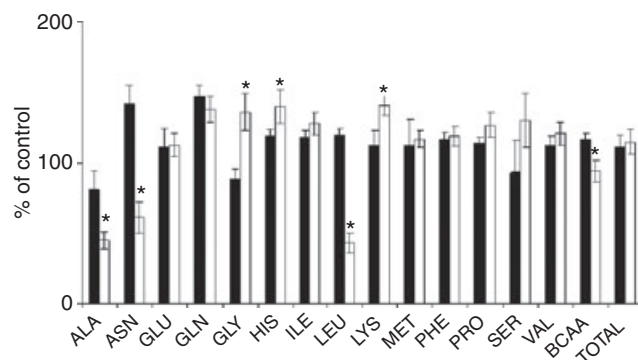


Figure 2 Relative effect of inflammation on amino acid release from EDL (filled bars) and SOL (open bars). Means of appropriate control values were used as 100%. Values are means ± SEM, n = 7 in each group. F-test followed by two-sample t-test; ^{*}P < 0.05 vs. EDL. SOL, the soleus muscle; EDL, the extensor digitorum longus muscle; BCAA, branched chain amino acids (valine, leucine, isoleucine); TOTAL, sum of all measured amino acids; ALA, ASN, GLU, GLN, GLY, HIS, ILE, LEU, LYS, MET, PHE, PRO, SER, VAL – standard amino acid abbreviations.

Discussion

We found that total proteolysis during inflammation increased comparably in SOL (28%) and EDL (34%), increase in myofibrillar proteolysis in SOL (24%) was lower than in EDL (56%). The differences in myofibrillar proteolysis could reflect the changes in CHTLA of proteasome which increased by 14% in SOL and 58% in EDL, while the total proteolysis could be influenced by other (e.g. lysosomal) mechanisms. Myofibrils cannot be cleaved by the proteasome itself (Salomon & Goldberg 1996). However, various catabolic conditions activate other proteases (e.g. caspase-3), which disintegrate myofibrils and make the actin and myosin accessible for proteasomal cleaving (Du *et al.* 2005). Our findings are in agreement with those of Hasselgren *et al.* (1989) who demonstrated that an increase in total and myofibrillar proteolysis during sepsis is significantly more pronounced in EDL than in SOL. Similar results were published by Tiao *et al.* (1997) and also after burn injury (Fang *et al.* 1998). We suggest that different response of EDL and SOL to inflammation could be caused by different sensitivities of white and red fibres to various humoral factors, e.g. hormones (Larbaud *et al.* 2001) and cytokines (Fang *et al.* 1995). In addition, glucocorticoids (Wang *et al.* 1998), proteolysis inducing factor-PIF (Lorite *et al.* 2001) and cytokines (Li *et al.* 1998) comprise some of the most potent substances which are able to affect the expression of genes of the ATP-dependent ubiquitin-proteasome system.

In agreement with the enhanced catabolic activity, the release of amino acids from muscle to incubation medium during inflammation tended to increase in both SOL and EDL. Considering the important role of leucine in muscle energy and protein metabolism, the relative changes in its release are very interesting. In SOL, the release of leucine decreased while in EDL, it increased in response to turpentine treatment, resulting in a significant difference. The decrease in leucine release in SOL was observed without a corresponding increase in leucine oxidation. We hypothesize that leucine accumulates in the muscle, and/or that its intermediate – α -ketoisocaproate – is more released into the medium or transformed to β -hydroxy- β -methylbutyrate. This suggestion is partly based on an observation of increased leucine intramuscular concentration after stress hormone administration (Hammarqvist *et al.* 2001).

In our study, we observed a significant difference between the decrease in protein synthesis in EDL (31%) and SOL (14%) following turpentine treatment. Our findings are in agreement with those of Vary & Kimball (1992), who observed that the protein synthetic rate in fast-twitch muscle was reduced by sepsis, whereas slow-twitch muscles were not affected. With regard to changes in CHTLA of proteasome

in SOL and EDL during inflammation, it may be suggested that the decrease in protein synthesis is related to the changes in activity of the proteasomal system. This suggestion is based on findings that some translation initiation factors, such as eIF4G or eIF3a are cleaved in the proteasome (Baugh & Pilipenko 2004). Moreover, as the removal of leucine reduces protein synthesis through changes in both eIF2B and eIF4E (Vary *et al.* 1999) and leucine is unique among the BCAA in its ability to stimulate protein synthesis in muscle (Anthony *et al.* 2000), we suggest that possible cumulation of leucine in SOL could affect protein synthesis.

Total proteolysis increased and leucine release decreased significantly while other parameters did not change in SOL of septic rats against control. EDL remained unaffected. As reported by Chai *et al.* (2002) significant increase in muscle proteolysis following burning was connected with rise of mRNA of several proteasomal subunits. Additionally, the proteasomal complex is highly stimulated by some cytokines. Chai *et al.* (2003) detected increases in proteasomal subunit expression and enhanced TNF- α and IL-6 levels after *Escherichia coli* endotoxin administration. Finally, Bazel *et al.* (1999) found, that CLP increases IL-6 levels in liver while s.c. turpentine application does not. With regard to protein synthesis, there is no agreement considering the effect of inflammation on skeletal muscle. Kadlcikova *et al.* (2004) observed decrease in protein synthesis in skeletal muscle after CLP while Hasselgren *et al.* (1986) and Wusteman *et al.* (1990) reported no significant changes in protein synthesis in skeletal muscle during sepsis after CLP or turpentine administration respectively.

We conclude that there are differences in protein and amino acid metabolism between SOL (slow, red muscle) and EDL (fast, white muscle) both in physiological conditions and after turpentine administration. The rate of catabolic and synthetic reactions is higher in SOL than in EDL. During inflammation, CHTLA of proteasome and protein synthesis are less affected in SOL compared to EDL, as well as the rate of release of leucine and some other amino acids.

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