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Increased muscle proteasome activities in rats fed a polyunsaturated fatty acid supplemented diet

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Abstract

Changes in the proteasome system, a dominant actor in protein degradation in eukaryotic cells, have been documented in a large number of physiological and pathological conditions. We investigated the influence of monounsaturated or polyunsaturated fatty acids (PUFAs) supplemented diets on the proteasome system, in rat skeletal muscles. Thirty rats were randomly assigned to three groups. The control group received only a standard diet. The monounsaturated fatty acid (MUFA) enriched diet group was fed with 3% sunflower oil in addition to standard food, and the polyunsaturated fatty acid supplemented diet group received 9% Maxepa® in addition to the standard diet. We analyzed muscle proteasome activities and content. Monounsaturated or PUFAs supplemented diets given for 8 weeks induced a significant increase in proteasome activities. With the polyunsaturated fatty acid enriched diet, the chymotrypsin-like and peptidylglutamylpeptide hydrolase activities increased by 45% in *soleus* and *extensor digitorum longus* (EDL), and by 90% in the gastrocnemius medialis (GM) muscle. Trypsin-like activity of the proteasome increased by 250% in soleus, EDL and GM. This increase in proteasome activities was associated with a concomitant enhancement in the muscle content of proteasome. Proteasome activities and level were less stimulated with a monounsaturated fatty acid supplemented diet. This study provides evidence that a monounsaturated or polyunsaturated fatty acid supplemented diet may regulate muscle proteasomes. Unsaturated fatty acids are particularly prone to free radical attack. Thus, we suggest that alterations in muscle proteasome may result from monounsaturated and polyunsaturated fatty acid-induced peroxidation, in order to eliminate damaged proteins.

Keywords: Proteolysis; Proteasome; Polyunsaturated fatty acids; Muscle

1. Introduction

Proteins in skeletal muscle undergo a continuous process of degradation and re-synthesis. Protein degradation is a complex set of interacting processes; three major proteolytic systems are well characterized in skeletal muscle: the lysosomal, the Ca^{2+}-dependent and the ubiquitin–proteasome pathways. Recent studies have shown that the bulk of overall and myofibrillar muscle protein breakdown

Abbreviations: AMC, 7-amido-4-methyl coumarin; β-NA, β-naphthylamide; ChT-L, chymotrypsin-like; DHA, docosahexaenoic acid; DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamide(4-guanidino)-butane; EDL, *extensor digitorum longus*; EPA, eicosapentaenoic acid; GM, *gastrocnemius medialis*; MUFA, monounsaturated fatty acid; PGPH, peptidylglutamyl peptide hydrolase; PUFA, polyunsaturated fatty acid; PVDF, polyvinylidene difluoride; T-L, trypsin-like

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is mediated by the ubiquitin–proteasome-dependent pathway and not by the lysosomal system (Furuno, Goodman, & Golberg, 1990). The former is the major system for protein degradation in eukaryotic cells (Attaix, Combaret, Kee, & Taillandier, in press). Changes in the expression of proteasome subunits and other proteins of the ubiquitination machinery have been reported in a large number of physiological and pathological conditions. A number of acute muscle wasting conditions are associated with an upregulation of the ubiquitin–proteasome system (Attaix et al., in press, 1994; Medina, Wing, & Goldberg, 1995; Mykles & Haire, 1991). Cancer cachexia is characterized by a rise in protein degradation. This increased protein catabolism can be suppressed by treating cancer mice with high levels of PUFAs (e.g. eicosapentaenoic acid (EPA)), which inhibited the muscle ubiquitin–proteasome pathway (Whitehouse, Smith, Drake, & Tisdale, 2001). A detergent effect of fatty acids as of SDS has also been demonstrated on the activities of purified proteasomes (Arribas & Castano, 1990; Dahlmann et al., 1993). Mild oxidative stress increases proteasome-dependent proteolysis. Davies (2001) have demonstrated that oxidized proteins are recognized and degraded by the 20S proteasome in the absence of ATP and ubiquitin, whereas 26S proteasome activity is inactivated.

The present study aimed to assess the influence of monounsaturated and PUFAs supplemented diets on the proteasome system. Thus, we have investigated proteasome peptidase activities in the muscles of Wistar rats fed with monounsaturated and ω-3 PUFAs enriched diets. We have measured the three best-characterized peptidase activities of the proteasome: chymotrypsin-like (ChT-L), trypsin-like (T-L) and peptidylglutamyl peptide hydrolase (PGPH) in three skeletal muscles owing to differences in their metabolic and contractile properties. The muscle content of 20S proteasomes was also determined by Western blot.

2. Materials and methods

Animals experiments were carried out in accordance with the "Guiding Principles for the Care and Use of Animals".

2.1. Animal/training protocol

Thirty growing male Wistar rats (3 months old) were obtained from the same breeding (IFFA-Credo, France). They were housed in a temperature controlled room at 20 ± 2 °C, 50 ± 10% hygrometry, with a 12 h day/night cycle.

Rats were divided randomly into three groups: a control group fed with a standard laboratory diet, a group fed with ω-3 PUFAs enriched diet and a third group fed with monounsaturated fatty acids (MUFAs) supplemented diet. The MUFA group was fed with 3% sunflower oil added to the standard food. The PUFA group received 9% Maxepa® added to the standard diet. "Maxepa®" is a natural fish flesh oil containing at least 30% ω-3 PUFAs including 18% eicosapentaenoic acid (EPA C20:5 *n*-3) and 12% docosahexaenoic acid (DHA C22:6 *n*-3). Clinical studies have shown that "Maxepa®" used daily reduced significantly triglyceride plasmatic concentrations, and thus is used for therapeutic indications (treatment of hyperglyceridemia). The Maxepa® supplement diet provided a 3% PUFA enriched diet. Animals were fed with these diets for 2 weeks before starting the experiment. Rats had free access to water and food. The composition of the standard, MUFA and PUFA enriched diets is given in Table 1.

Rats were weighed daily. After 8 weeks of experimentation, rats fasted for 12 h were anaesthetized by an intra-peritoneal injection of pentobarbital sodium, and sacrificed by cardiac puncture. Soleus, EDL and GM muscles were removed, weighed and frozen in liquid nitrogen and used immediately for protein and enzyme assays and Western blot analysis.

2.2. Preparation of muscle extracts

Extracts were prepared according to Solomon and Goldberg (1996) with slight modifications. Soleus, EDL and GM muscles were placed in 50 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM E64 (*trans*-epoxysuccinyl-L-leucylamide(4-guanidino)-butane), 2.5 μM pepstatin A and homogenized with a Polytron. The homogenates were centrifuged for 1 h at 100,000 × *g*, 4 °C. The supernatants were collected and used for protein and enzyme assays and for Western blot

Table 1
Composition of the standard, MUFA and ω-3 PUFA supplemented diets

	Standard diet	MUFA enriched diet	ω-3 PUFA enriched diet
Cereals mix (g/kg diet)	839	814	764
Vitamins and minerals mix (g/kg diet)	41	40	37
Animal proteins (g/kg diet)	40	39	36
Vegetal proteins (g/kg diet)	80	77	73
Oil (g/kg diet)	–	30	–
Maxepa® (g/kg diet)	–	–	90
Carbohydrate (% kcal)	76		74
Protein (% kcal)	20		19
Fat (% kcal)	4		7
Energy value (kcal/g diet)	2.9	3.2	3.5
Fatty acids (mg/kg diet)			
Palmitic acid	4000		3640
Palmitoleic acid	600		546
Stearic acid	Traces		Traces
Oleic acid	6400		5824
Linoleic acid	12400		11284
Linolenic acid	90		82
Eicosapentaenoic acid			1620
Docosahexaenoic acid			1080
Total fatty acids (%)			
Saturated fatty acids	20	10	17
Monounsaturated fatty acids	27	48	24
Polyunsaturated fatty acids	53	42	59

analysis. The experimental conditions (pH 8.0, presence of EDTA, EGTA and protease inhibitors) allows direct measurement of proteasome activities in the crude extract, without interference from other known proteolytic systems (Farout et al., 2000). Protein concentration was determined by the Bradford method using the Biorad assay reagent with bovine serum albumin used as a standard (Bradford, 1976).

2.3. Peptidase activities

All assays were done in triplicate. Reaction mixtures (200 μl) contained 50 mM Tris–HCl buffer (pH 8.0), 1 mM dithiothreitol (DTT), 40–100 μg protein and suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (40 μM) or Nt-boc-Leu-Ser-Thr-Arg-7-amino-4-methyl coumarin (40 μM) or Cbz-Leu-Leu-Glu-β-naphthylamide (100 μM) (Sigma). After incubation for 30 min at 37 °C, the reaction was stopped by adding 800 μl of 100 mM monochloroacetate–30 mM sodium acetate. Fluorescence was monitored on a Hitachi F-2000 fluorimeter (excitation 370 nm, emission 430 nm for 7-amino-4-methyl coumarin (AMC) substrates and excitation 333 nm, emission 410 nm for β-naphthylamide (β-NA) substrates).

2.4. Western blot analysis

Immunoelectrophoretic blot analysis was carried out by the method of Towbin, Staehelin, and Gordon (1976). Samples (10 μg of protein) were separated by SDS-PAGE according to Laemmli (1970) and transferred electrophoretically to polyvinylidene difluoride (PVDF) sheets (Immobilon™-P, Millipore). The membranes were saturated with 10% skimmed milk, 0.1% Tween in TBS buffer for 2 h at room temperature before incubation for 1 h at 37 °C with the mouse monoclonal anti-alpha 1 antibody diluted to 1/4000 (Cappel), used as the primary antibody. After washing in TBS-0.1% Tween, the second antibody coupled to peroxidase was added for 1 h at room temperature. Membranes were revealed by chemiluminescence

using the ECL™ kit (Amersham) and exposed to film (Cronex 5, Agfa). Signal intensity of the developed film was quantified densitometrically and analyzed using the Biorad image analysis software (Quantity One, Biorad). An identical reference consisting of a muscle extract was loaded on each blot. Samples are compared with the reference on the same blot. Final data are expressed in percentage of the standard fed group. We have verified that the intensity of the signals is proportional to the amount of protein loaded on gels.

2.5. Statistical analysis

Results are given as mean ± standard error of the mean. The data were compared by ANOVA followed by Fisher's test. The significance level was set at $P \leq 0.05$.

3. Results

3.1. Muscle and body mass

Although the standard, MUFA and ω-3 PUFA enriched diets provided different amount of energy, the energy intake of rats was similar (control group: 72 ± 2.3 kcal per day; MUFA fed group: 72 ± 3.6 kcal per day; and ω-3 fed group: 71.8 ± 1.6 kcal per day). Whole body weight and relative muscle mass to body mass, were not significantly different in the three groups (Table 2).

Table 2
Body mass and muscle mass relative to body mass of rats with different diets

	Standard diet	MUFA enriched diet	ω-3 PUFA enriched diet
Body mass (g)	493 ± 10	525 ± 10	523 ± 11
Relative muscle mass (mg/g)			
Soleus	0.34 ± 0.01	0.38 ± 0.01	0.37 ± 0.01
EDL	0.42 ± 0.01	0.44 ± 0.01	0.41 ± 0.01
GM	2.15 ± 0.04	2.29 ± 0.04	2.11 ± 0.04

Results are means ± S.E.M., $n = 15$. There was no significant difference between groups ($P \leq 0.05$).

3.2. Peptidase activities

ChT-L, T-L and PGPH proteasomal activities increased in all skeletal muscles of rats fed with ω-3 PUFA or MUFA supplemented diets compared to control rats, indicating a possible increase in the capacity to perform protein breakdown. For ω-3 PUFA enriched diet, ChT-L and PGPH peptidase activities increased by about 45% in soleus and EDL. From rats fed the ω-3 PUFA enriched diet the T-L activity increased by 229% in soleus and by 134% in EDL. Activities increased by 250% in GM muscle. The MUFA supplemented diet, also stimulated proteasomal activities, but to a lesser extent (Table 3).

Table 3
The effect of MUFA and ω-3 PUFA enriched diets on muscle proteasomal activities

		ChT-L	T-L	PGPH
Soleus	Control	6.2 ± 0.46	1.17 ± 0.06	10.38 ± 0.59
	MUFA enriched diet	7.3 ± 0.38*	1.56 ± 0.09	13.39 ± 0.93*
	ω-3 enriched diet	8.8 ± 0.39*,#	3.78 ± 0.17*,#	15.12 ± 0.66*
EDL	Control	3.72 ± 0.32	0.63 ± 0.06	5.69 ± 0.91
	MUFA enriched diet	4.00 ± 0.39	1.36 ± 0.05*	7.33 ± 0.48
	ω-3 enriched diet	4.98 ± 0.45*,#	1.48 ± 0.11*	8.36 ± 1.26
GM	Control	4.87 ± 0.31	0.86 ± 0.01	9.40 ± 0.33
	MUFA enriched diet	7.80 ± 0.54*	1.82 ± 0.04*	13.04 ± 0.91*
	ω-3 enriched diet	9.20 ± 0.34*,#	3.01 ± 0.10*,#	17.74 ± 0.39*,#

Muscle extracts were assayed for the ChT-L, T-L and PGPH activities. Activities are expressed as nmol/30 min mg protein. Values are means ± S.E.M., $n = 5–7$.

* Significantly ($P \leq 0.05$) different from control.

Significantly ($P \leq 0.05$) different from MUFA enriched diet.

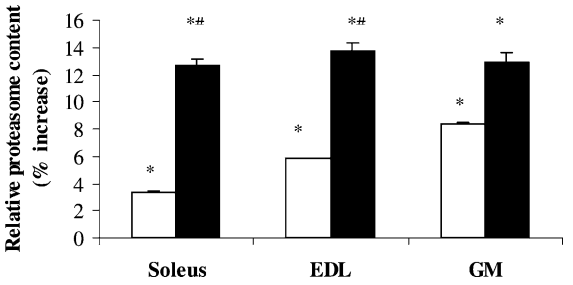

Fig. 1. Incidence of MUFA and ω-3 PUFA enriched diets on proteasome level in rat skeletal muscle. The intramuscular protein of subunit α1 of the 20S proteasome were analyzed by Western blotting followed by densitometric quantification, means ± S.E.M., $n = 5–7$. Data are expressed in percentage increase vs. the standard fed group ((□) MUFA enriched diet, (■) ω-3 enriched diet).

3.3. Western blot analysis

Western blot analysis was performed by using an antibody directed against the proteasome α1 subunit, which is indicative of the muscle 20S proteasome levels. The ω-3 enriched diet increased proteasome levels in soleus, EDL and GM by 13% (Fig. 1). There was a small increase in muscle proteasome levels with the MUFA supplemented diet. Overall changes in proteasome levels occurred in parallel with changes in proteasome activities.

4. Discussion

Like all eukaryotic cells, skeletal muscle contains multiple proteolytic systems that include the lysosomal, Ca^{2+}-dependent (e.g. calpains I and II), and ATP–ubiquitin–proteasome-dependent pathways. Inhibitors of the lysosomal and Ca^{2+}-dependent systems slightly reduce overall increased proteolysis and do not block the enhanced breakdown of myofibrillar proteins in incubated muscles from catabolic rodents (Attaix et al., 1994; Attaix & Taillandier, 1998; Furuno et al., 1990; Medina et al., 1995; Whitehouse et al., 2001; Wing, Haas, & Goldberg, 1995). In contrast, the activation of the ATP–ubiquitin–proteasome-dependent is responsible for most stimulated muscle proteolysis and for the breakdown of myofibrillar proteins (Attaix et al., in press, 1994; Attaix & Taillandier, 1998; Furuno et al., 1990; Medina et al., 1995; Whitehouse et al., 2001; Wing et al., 1995). Accordingly, ubiquitin–proteasome-dependent proteolysis, but not cathepsins and/or calpains, is systematically activated in the muscles from cachectic animals (Attaix & Taillandier, 1998).

In the present study, we investigated the effect of MUFA and ω-3 PUFA supplemented diets on muscle proteasome-dependent proteolysis. Measurements of proteasome activities and level were performed on three skeletal muscles soleus, EDL and GM owing to their different metabolic and contractile properties. The slow-twitch red fiber soleus is mainly composed of type I fibers (87%), which exhibit an oxidative metabolism. The fast-twitch EDL is mostly composed of type II fibers adapted to anaerobic glycolytic metabolism. Finally, the GM possesses mixed glycolytic/oxidative fibers and is adapted to both aerobic and anaerobic metabolism (Garlick, Maltin, Bailli, Delday, & Grubb, 1989).

The major observation of this study is that muscle proteasome activities increased with a diet moderately supplemented with ω-3 PUFA. This suggests that changes in the diet may confer to muscle cells a greater capacity to degrade proteins. A MUFA enriched diet had a similar, but markedly attenuated effect. The effect of MUFA and ω-3 PUFA enriched diets might be explained by their different fatty acid composition. There is no accumulation of triglycerides in skeletal muscle with the diets used (Clavel, Farout, Briand, Briand, & Jouanel, 2002). The lack of changes in whole-body mass and relative muscle mass, and the stimulation of proteasome-dependent proteolysis suggests that protein synthesis was also increased in skeletal muscle. MUFA and ω-3 PUFA enriched diets may induce changes in metabolism. MUFA and PUFA are particularly prone to free radical attack because the presence of a double bond weakens the carbon–hydrogen bond at the adjacent carbon atom. Peroxidation generates radicals, hydroperoxides and other reactive species (Foretz, Foufelle, & Ferre, 1999). Conceivably, an enhanced production of such molecules can potentially damage different macromolecules, including proteins, which in turn may result in cellular degeneration. In addition, PUFA up-regulated the expression of genes encoding proteins involved in fatty acid oxidation via activation of peroxisome proliferator-activated receptor a (Keller et al., 1993). This may explain increased proteasome activities in order to eliminate damaged proteins. Davies (2001) have demonstrated

that the core 20S proteasome is responsible for the turnover of oxidatively modified proteins in a ATP- and ubiquitin-independent process.

To investigate whether increased proteasome activities reflected a parallel increase in the muscle content of 20S proteasomes, we measured 20S proteasome levels using an antibody against the α1 proteasome subunit. The administration of the ω-3 PUFA enriched diet resulted in an increase in α1 subunit content, in all muscles studied. This suggests that the ω-3 PUFA supplemented diet stimulated both proteasome activities and levels to cope with damaged proteins resulting from PUFA peroxidation. In contrast (Whitehouse & Tisdale, 2001; Whitehouse et al., 2001) have demonstrated that in cancer cachexia and starvation, eicosapentaenoic acid attenuated loss of body weight and suppressed muscle protein catabolism mediated by the proteasome. This effect was not observed with docosahexaenoic acid or linoleic acid and may be related to the ability to suppress 15-hydroxyeicosatetraenoic acid formation. However, these experiments were conducted in the presence of very high levels of PUFA. In our experimental conditions, rats were fed with a moderately ω-3 PUFA supplemented diet.

In conclusion, a moderately ω-3 PUFA enriched diet stimulated both proteasome activities and proteasome levels in three rat skeletal muscles soleus, EDL and GM. Sugihara, Tsuruta, Date, Furuno, and Kohashi (1994) have shown a high susceptibility of PUFA to peroxidation. Peroxidation products are deleterious to the cell and can cause damaged proteins. We suggest that increases in proteasome activities and levels might be necessary to eliminate damaged proteins. The adaptations in proteasome activities and levels were also observed with a MUFA enriched diet, but to a lesser extend. This diet with high oleic content has an unsaponifiable fraction rich in antioxidants, including phenolic compounds. These antioxidative properties reduced the susceptibility to lipid peroxidation (Ochoa, Quiles, Ramirez-Tortosa, Mataix, & Huertas, 2002), and MUFA exhibit a better resistance to oxidation than PUFA.

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