A leucine-supplemented diet restores the defective postprandial inhibition of proteasome-dependent proteolysis in aged rat skeletal muscle

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We tested the hypothesis that skeletal muscle ubiquitin–proteasome-dependent proteolysis is dysregulated in ageing in response to feeding. In Experiment 1 we measured rates of proteasome-dependent proteolysis in incubated muscles from 8- and 22-month-old rats, proteasome activities, and rates of ubiquitination, in the postprandial and postabsorptive states. Peptidase activities of the proteasome decreased in the postabsorptive state in 22-month-old rats compared with 8-month-old animals, while the rate of ubiquitination was not altered. Furthermore, the down-regulation of *in vitro* proteasome-dependent proteolysis that prevailed in the postprandial state in 8-month-old rats was defective in 22-month-old rats. Next, we tested the hypothesis that the ingestion of a 5% leucine-supplemented diet may correct this defect. Leucine supplementation restored the postprandial inhibition of *in vitro* proteasome-dependent proteolysis in 22-month-old animals, by down-regulating both rates of ubiquitination and proteasome activities. In Experiment 2, we verified that dietary leucine supplementation had long-lasting effects by comparing 8- and 22-month-old rats that were fed either a leucine-supplemented diet or an alanine-supplemented diet for 10 days. The inhibited *in vitro* proteolysis was maintained in the postprandial state in the 22-month-old rats fed the leucine-supplemented diet. Moreover, elevated mRNA levels for ubiquitin, 14-kDa ubiquitin-conjugating enzyme E2, and C2 and X subunits of the 20S proteasome that were characteristic of aged muscle were totally suppressed in 22-month-old animals chronically fed the leucine-supplemented diet, demonstrating an *in vivo* effect. Thus the defective postprandial down-regulation of *in vitro* proteasome-dependent proteolysis in 22-month-old rats was restored in animals chronically fed a leucine-supplemented diet.

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Ageing is characterized by a gradual loss of muscle proteins (sarcopenia), which is ultimately responsible for decreased mobility and autonomy. Sarcopenia also reduces the ability of the elderly to cope with nutritional, infectious or traumatic stresses, whose incidence increases in ageing (Young *et al.* 1989). Proteins in skeletal muscle, as in other mammalian tissues, undergo a continuous process of synthesis and degradation (Waterlow *et al.* 1978). Thus, sarcopenia should be due to an imbalance between rates of protein turnover. Some studies demonstrated an age-related decline in synthesis rate of mixed muscle proteins, myosin heavy chain and mitochondrial proteins (Rooyackers *et al.* 1996; Greenlund & Nair, 2003) although these findings have been challenged (Volpi *et al.* 2001).

Skeletal muscle protein synthesis decreases in the postabsorptive (PA) state and increases in the postprandial (PP) state, while protein breakdown follows the inverse pattern. In adults, net positive protein balance in the PP state and net negative protein balance in the PA state cancel each other. In humans protein mass increases in daytime and decreases overnight so that muscle protein mass does not change throughout the day and night cycle. There is strong evidence that the stimulatory effect of amino acids on protein synthesis is blunted in old muscles from both animals (Mosoni *et al.* 1995; Dardevet *et al.* 2000; Arnal *et al.* 2002) and humans (Guillet *et al.* 2004; Cuthbertson *et al.* 2005). A limited number of studies also suggest that

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there are alterations in muscle proteolysis in ageing in either the PA state in humans (Volpi *et al.* 2001) or the PP state in rats (Arnal *et al.* 2002). Similar observations have been reported in humans at the whole-body level in the PP state (Arnal *et al.* 1999). Changes in protein turnover rates in response to feeding are mediated by both hormones and nutrients. Among them leucine stimulates protein synthesis in incubated skeletal muscles from adult rats as all (Dardevet *et al.* 2000) or branched-chain (Anthony *et al.* 2000*a*) amino acids do, by enhancing translation initiation (Dardevet *et al.* 2000; Anthony *et al.* 2000*a*, *b*; Greiwe *et al.* 2001) via a rapamycin-sensitive pathway (Dardevet *et al.* 2000; Anthony *et al.* 2000*a*). Essential amino acids are primarily responsible for the meal-induced stimulation of muscle protein anabolism in the elderly (Volpi *et al.* 2003). Accordingly, *in vivo* a leucine-supplemented meal corrects the defective postprandial stimulation of muscle protein synthesis in old rats (Dardevet *et al.* 2002). Leucine also inhibits whole-body protein degradation *in vivo* (Frexes-Steed *et al.* 1992) and skeletal muscle proteolysis *in vitro* (Buse & Reid, 1975; Fulks *et al.* 1975; Tischler *et al.* 1982). However, to our knowledge, whether leucine has a possible inhibitory role on muscle proteolysis in the PP state is unknown.

Multiple proteolytic pathways, including the lysosomal, Ca^{2+}-dependent, ubiquitin (Ub)–proteasome-dependent processes, and proteases (e.g. caspases and matrix metallo-proteases) are responsible for skeletal muscle proteolysis (Attaix *et al.* 2003). The Ub–proteasome-dependent pathway degrades the bulk of muscle proteins including myofibrillar components (Jagoe & Goldberg, 2001; Hasselgren & Fischer, 2001; Attaix *et al.* 2003). In this pathway, Ub targets specific intracellular proteins for degradation. Briefly, Ub conjugation proceeds via a thiol ester reaction cascade involving the Ub-activating enzyme (E1), Ub-conjugating enzymes (E2), and Ub-protein ligases (E3), which possess substrate recognition sites (for recent reviews see Pickart, 2001; Glickman & Ciechanover, 2002). Rapid degradation by the 26S proteasome requires the formation of a polyUb degradation signal that comprises at least four Ub moieties (Pickart, 2001).

Information on the regulation of muscle Ub–proteasome-dependent proteolysis in ageing and in response to feeding is very limited. The aim of the present study was to investigate whether (i) the muscle Ub–proteasome system is regulated in the PP state in both adult and old rats, and (ii) leucine supplementation in the diet affects this response. We report that an inhibition of Ub–proteasome-dependent proteolysis prevails in the PP state in 8-month-old rats, but is lacking in 22-month-old animals. We also demonstrate that leucine supplementation in the diet completely restores this defect in 22-month-old rats by regulating both proteasome activities and rates of substrate ubiquitination. Finally, we provide evidence that chronic leucine supplementation in the diet has long-lasting effects on the muscle Ub–proteasome system from 22-month-old rats.

Methods

Animals and experimental design

The experiments were conducted in accordance with the French National Research Council's Guidelines for the Care and Use of Laboratory Animals. Eight- and 22-month-old Male Wistar rats (Iffa Credo, Lyon, France) were used. The experimental design has been previously described in detail (Dardevet *et al.* 2002; Rieu *et al.* 2003). In brief, rats were fed a standard diet during the 8-h dark period for 1 month. In Experiment 1, the effect of a unique meal supplemented with 5% of leucine was tested against a meal supplemented with 5% alanine (controls), so that the rats were fed the same amount of protein and energy. Over the 1-h feeding period, food intake was not affected by the meal consumed (Dardevet *et al.* 2002). In Experiment 2, diets were tested for 10 days. Rats daily received either the leucine-supplemented or the alanine-supplemented meal for the first hour of feeding followed by the standard diet for the next 7 h. Experimental and control diet daily intakes were similar in both 8- and 22-month-old rats during the first 9 days of the experimental period. On the day of the experiment, rats in the PA state were overnight fasted, whereas rats in the PP state consumed the same amount of food as on the previous days and were studied 2 h after the first meal was given (Rieu *et al.* 2003). In both experiments rats were anaesthetised with sodium pentobarbital (45 μg/g body weight) and killed by overdose of the anaesthetic.

Measurements of *in vitro* rates of proteolysis

Epitrochlearis muscles from rats in Experiment 1 or 2 were quickly excised and rinsed in Krebs-Henseleit bicarbonate buffer ((mM) NaCl 120, KCl 4.8, $NaHCO_3$ 25, KH_2PO_4 1.2 and $MgSO_4$ 1.2, pH 7.4), supplemented with 5 mM Hepes, 5 mM glucose and 0.1% BSA. Muscles were then transferred to plastic tubes containing 1.5 ml of fresh buffer saturated with a 95% O_2–5% CO_2 gas mixture. After 30 min of preincubation, muscles were transferred to a fresh medium of identical composition and further incubated for 1 h.

Rates of protein breakdown were measured by following the rates of tyrosine release into the medium in the presence of 0.5 mM cycloheximide, which blocks protein synthesis. Non-lysosomal and Ca^{2+}-independent proteolysis was measured in a Ca^{2+}-deprived medium supplemented with 50 μM leupeptin and 10 mM methylamine (Combaret *et al.* 2004). Proteasome-dependent proteolysis was calculated as the difference between the rates of non-lysosomal and Ca^{2+}-independent proteolysis (measured in one muscle)

and the rates of non-lysosomal, Ca^{2+}-independent and proteasome-independent proteolysis (measured in the contralateral muscle) following incubation with 50 μM leupeptin, 10 mM methylamine and 40 μM MG132 in a Ca^{2+}-deprived medium (Combaret *et al.* 2004). Protein degradation was expressed in nanomoles of tyrosine released in the medium per milligram protein per hour. Muscle protein content was measured according to the bicinchoninic acid procedure.

Peptidase activities of the proteasome

Proteins from pooled extensor digitorum longus (EDL) muscles of 8- and 22-month-old animals in the PA or PP states (Experiment 1) were homogenized in ice-cold buffer (pH 7.5) containing 50 mM Tris, 250 mM sucrose, 10 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors (10 μg ml^{-1} of antipain, aprotinin, leupeptin and pepstatin A, and 20 μM PMSF). The proteasomes were isolated by three sequential centrifugations as described previously (Hobler *et al.* 1999; Fang *et al.* 2000). The final pellet was resuspended in buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 20% glycerol. The protein content of the proteasome preparation was determined according to Lowry *et al.* (1951). Peptidase activities of the proteasome were determined by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY), Boc-Leu-Arg-Arg-7-amido-4-methylcoumarin (LRR) and the Cbz-Leu-Leu-Glu-β-naphtylamide (LLE) (Sigma). These substrates are preferentially hydrolysed by the chymotrypsin-like, the trypsin-like and the peptidyl glutamyl peptide hydrolase (PGPH) activities of the proteasome, respectively (Craiu *et al.* 1997; Hobler *et al.* 1999). To measure peptidase activity, 10 μl of the proteasome extract was added to 40 μl of medium containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 2 U apyrase and 300 μM of LLVY, or 800 μM of LRR or LLE. The peptidase activity was determined by measuring the accumulation of the fluorogenic cleavage product (methylcoumaryl-amide, AMC or β-naphtylamide, β-Na) using a luminescence spectrometer LS50B (Perkin Elmer). Fluorescence was measured every 5 min at 37°C during 45 min at 380 nm (AMC) or 333 nm (β-Na) excitation wavelength, and 440 nm (AMC) or 450 nm (β-Na) emission wavelength. The difference between arbitrary fluorescence units recorded with or without 40 μM of the proteasome inhibitor MG132 (Affiniti) in the reaction medium was calculated, and the final data were corrected by the amount of protein in the reaction. The time course for the accumulation of AMC or β-Na after hydrolysis of the substrate was analysed by linear regression to calculate peptidase activities, e.g. the slopes of best fit of accumulated AMC or β-Na *versus* time. Different kinetics were performed to measure individually the three proteasome peptidase activities.

Ubiquitination rates

EDL muscles (Experiment 1) from the same group of rats were pooled and homogenized at 4°C with a Polytron in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF, 10 μg ml^{-1} pepstatin A and 10 μg ml^{-1} leupeptin (5 ml buffer (g muscle)$^{-1}$). The homogenates were centrifuged (10 000 *g*, 10 min, 4°C). The resulting supernatants were centrifuged at 100 000 *g* for 60 min at 4°C. The final supernatants were stored at −80°C until use. Rates of ubiquitination were determined by incubation at 37°C of muscle extracts containing 50 μg of protein in 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 2 mM 5′-adenylylimidodiphosphate and 5 μM [^{125}I]labelled Ub (~3000 cpm (pmol)$^{-1}$) in a total volume of 20 μl. The reaction was stopped at 0, 3, 6, 9 and 12 min by the addition of Laemmli buffer 1×. Conjugation rates were linear for this period of time and the concentration of exogenously labelled Ub was in significant excess of any endogenous Ub (e.g. addition of larger amounts of labelled Ub did not increase rates of ubiquitination) (Kee *et al.* 2003; Combaret *et al.* 2004). After incubation, Ub conjugates were resolved from free Ub by SDS–PAGE on 12% gels. After drying of the gel, high molecular weight radiolabelled conjugates were visualized with a Phosphofluoro-Imager (Molecular Dynamics), excised and the [^{125}I]Ub bound to protein substrates was determined by measuring the radioactivity in a Cobra II auto-gamma counter (Packard). The time course for the accumulation of high molecular weight conjugates was analysed by linear regression to calculate ubiquitination rates, e.g. the slopes of best fit of cpm bound to Ub conjugates *versus* time.

Northern blots

EDL muscles of animals from Experiment 2 were rapidly excised, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted (Chomczynski & Sacchi, 1987), and Northern blots were performed as previously described (Combaret *et al.* 2004). The membranes were hybridized with cDNA probes encoding polyUb, the 14-kDa E2, and the C2 and X subunits of the 20S proteasome (Mansoor *et al.* 1996; Tilignac *et al.* 2002; Combaret *et al.* 2004). The hybridizations were performed with [^{32}P] cDNA fragments labelled by random priming. After stripping of the different probes, the filters were reprobed with a cDNA fragment encoding the 18S rRNA. The filters were autoradiographed, and the signals were quantified as previously described (Deval *et al.* 2001). Autoradiographic signals were

Table 1. Body weights and muscle masses of 8- and 22-month-old rats in Experiments 1 and 2

Experiment 1	8-month-old	22-month-old
n	29	31
Body weight (g)	598 ± 6^{A}	608 ± 10^{A}
Muscle mass (g (100 g BW)$^{-1}$)		
Tibialis anterior	165 ± 3^{A}	150 ± 3^{B}
EDL	40 ± 1^{A}	40 ± 1^{A}
Epitrochlearis	23 ± 1^{A}	23 ± 1^{A}

	8-month-old		22-month-old	
Experiment 2	Ala	Leu	Ala	Leu
n	20	20	22	22
Final body weight (g)	523 ± 8^{A}	533 ± 7^{AB}	567 ± 14^{BC}	572 ± 17^{C}
Muscle mass (g (100 g BW)$^{-1}$)				
Tibialis anterior	178 ± 3^{A}	173 ± 3^{A}	164 ± 4^{B}	161 ± 3^{B}
EDL	42 ± 1^{A}	41 ± 1^{A}	41 ± 1^{A}	41 ± 1^{A}
Epitrochlearis	26 ± 1^{A}	25 ± 1^{A}	24 ± 1^{A}	26 ± 1^{A}

Values are means ± S.E.M. for the number of rats indicated (*n*). Different superscript letters within a line indicated significant differences by ANOVA ($P < 0.05$).

normalized using the corresponding 18S rRNA signals to correct for variations in RNA loading.

Statistical analysis

All data are expressed as means ± S.E.M. Statistical analyses were performed using ANOVA or the unpaired Student's *t* test, as appropriate. If statistically significant differences were detected by ANOVA, *post hoc* comparisons between groups were made using the Fisher's PLSD test. Differences in proteasome activities and ubiquitination rates were assessed by comparing the slopes of best fit obtained by regression analysis (Kee *et al.* 2003). Significance was defined at the 0.05 level.

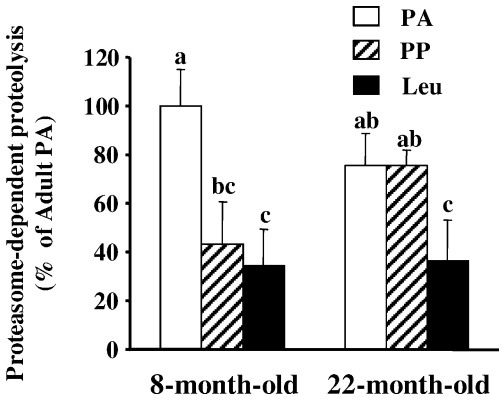

Figure 1. Skeletal muscle proteasome-dependent proteolysis after feeding 8- and 22-month-old animals
Rats from Experiment 1 were overnight starved (PA), or fed during 1 h either a alanine-supplemented (PP) or a leucine-supplemented meal (Leu). Proteasome-dependent proteolysis was measured as indicated in Methods in incubated epitrochlearis muscles harvested in the PA state or 2 h after meal ingestion. Values are means ± S.E.M. (vertical bars) for 9–10 animals and are expressed as a percentage of 8-month-old PA. Columns with different letters are significantly different from each other as assessed by ANOVA ($P < 0.05$).

Results

Body weights and muscle masses

Body weights of 8- and 22-month-old rats were similar in Experiment 1 (Table 1). In Experiment 2 the body weights of the 22-month-old-rats were slightly higher than in their 8-month-old respective controls (Table 1). In both Experiments 1 and 2, an atrophy of the tibialis anterior muscle (and of the gastrocnemius, data not shown) prevailed in 22-month-old rats, compared with the respective 8-month-old animals, but was not observed in the EDL and epitrochlearis muscles (Table 1).

Ubiquitin–proteasome-dependent proteolysis in the postprandial and postabsorptive states

Figure 1 shows that *in vitro* proteasome-dependent proteolysis measured in the absence and presence of the proteasome inhibitor MG132 decreased by 56% ($P < 0.05$) after feeding 8-month-old rats a normal diet, but was not down-regulated in 22-month-old animals.

There are two major steps in the Ub–proteasome system: (i) the labelling of substrates by a poly Ub degradation signal, and (ii) the subsequent breakdown of the targeted protein by the proteasome (Pickart, 2001; Glickman & Ciechanover, 2002). Thus, we measured ubiquitination rates and major proteasome peptidase activities. The PP inhibition of the Ub–proteasome system

in the muscle of the 8-month-old rats was due to a 41–45% decrease in chymotrypsin-, trypsin-like and PGPH proteasome activities (Fig. 2, and data not shown for the PGPH activity), without any change in rates of ubiquitination (Fig. 3). By surprising contrast, muscle proteasome activities slightly increased following meal ingestion in the 22-month-old rats (Fig. 2).

Effects of ageing on ubiquitin–proteasome-dependent proteolysis

Rates of proteasome-dependent proteolysis with ageing tended to decrease in the PA state and to increase in the PP state, but these variations were not significant (Fig. 1). By contrast, there were significant decreases in both chymotrypsin-like and trypsin-like proteasome activities in the PA state with ageing (Fig. 2). Only the trypsin-like proteasome activity increased with ageing in the PP state (Fig. 2), and the rate of ubiquitination was unaffected by ageing in both the PA and PP states (Fig. 3).

Effects of a single leucine-supplemented meal on ubiquitin–proteasome-dependent proteolysis

A full stimulation of skeletal muscle protein synthesis in the PP state was restored in 22-month-old rats fed a leucine-supplemented diet (Dardevet *et al.* 2002). This prompted us to investigate whether leucine may also inhibit muscle proteolysis. Figure 1 shows that a leucine-supplemented diet had no additive effect with feeding in 8-month-old rats. Accordingly, neither proteasome activities (Fig. 2) nor rates of ubiquitination (Fig. 3) were affected in such conditions. By contrast, the leucine-supplemented diet fully restored the defective postprandial inhibition of *in vitro* proteasome-dependent proteolysis in 22-month-old rats (Fig. 1). Increased proteasomal activities observed after feeding 22-month-old rats a normal diet were completely

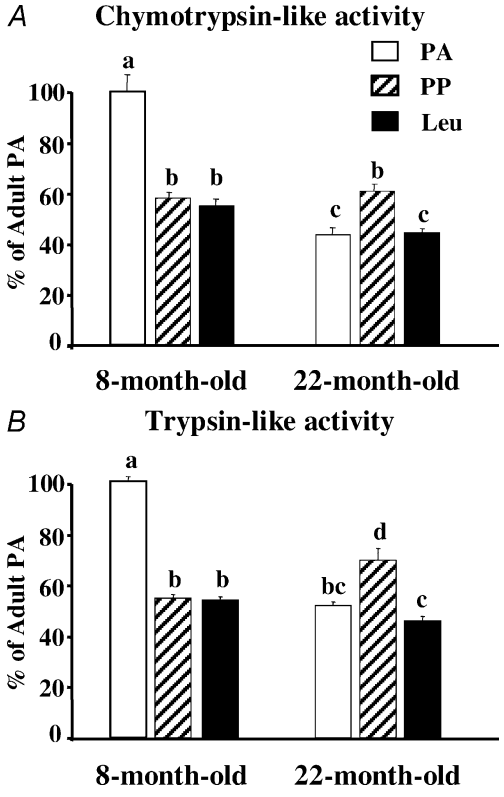

Figure 2. Chymotrypsin-like (*A*), and trypsin-like (*B*) peptidase activities of the proteasome in pooled extensor digitorum longus muscles ($n = 7$–10 rats) from 8- and 22-month-old rats
Animals from Experiment 1 were overnight starved (PA) or fed during 1 h (PP or Leu) as described in Fig. 1 legend. Data represent the slopes of best fit of arbitrary fluorescence units released from Suc-LLVY-AMC (chymotrypsin-like activity) or Boc-LRR-AMC (trypsin-like activity) *versus* time. Data are expressed as percentage of 8-month-old PA and bars denote standard errors of the slopes. Columns with different letters are significantly different by comparing the slopes of best fit ($P < 0.05$).

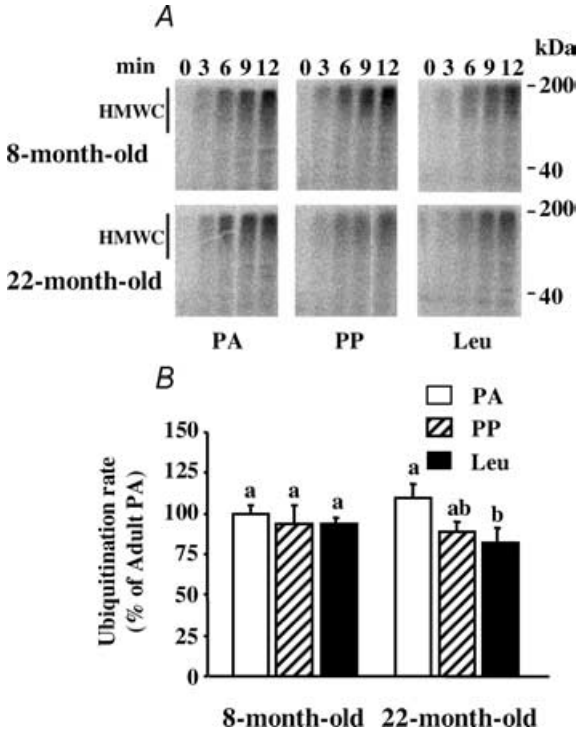

Figure 3. Ubiquitination rates in pooled extensor digitorum longus muscles ($n = 9$–11 rats) from 8- and 22-month-old rats
The formation of high molecular weight [^{125}I]Ub conjugates (HMWC) in extensor digitorum longus muscle extracts was followed by autoradiography (*A*). Animals from Experiment 1 were overnight starved (PA) or fed during 1 h (PP or Leu) as described in Fig. 1 legend. *B*, the comparison of ubiquitination rates (e.g. the slopes of best fit of cpm bound to HMWC following gel excision *versus* time). Data are expressed as percentage of 8-month-old PA and bars denote standard errors of the slopes. Columns with different letters are significantly different by comparing the slopes of best fit ($P < 0.05$).

prevented by leucine supplementation (Fig. 2), and rates of ubiquitination also decreased (−25%, $P < 0.05$; Fig. 3).

Effects of feeding a leucine-supplemented diet for 10 days on ubiquitin–proteasome-dependent proteolysis

Since leucine-supplemented meal feeding for 10 days had beneficial effects on muscle protein synthesis in 22-month-old rats (Rieu *et al.* 2003), we next investigated if proteolysis was also regulated long-term by leucine. Figure 4 shows that overall *in vitro* muscle proteolysis was inhibited in the PP state in 22-month-old animals chronically fed a leucine-supplemented diet compared with animals chronically fed an alanine-enriched diet. The leucine-supplemented diet inhibited a non-lysosomal Ca^{2+}-independent proteolytic process since the PP inhibition of *in vitro* muscle proteolysis was still detected in the presence of inhibitors of both cathepsins and calpains (Fig. 4).

As these experiments were performed with incubated muscles and may not reflect the *in vivo* situation, we next measured mRNA levels for components of the Ub–proteasome system in both 8- and 22-month-old rats. The mRNA levels for Ub, the 14-kDa E2, and the non-catalytic C2 or catalytic X subunit of the 20S proteasome increased in 22-month-old rats compared with 8-month-old animals (Fig. 5*A*).

Supplementation of the diet with leucine for 10 days completely suppressed the increased muscle mRNA levels for Ub, the 14-kDa E2, and C2 or X proteasome subunits in 22-month-old rats (Fig. 5*B*). These data demonstrate that chronic administration of a leucine-supplemented diet has long-lasting effects on the Ub-proteasome system *in vivo*, and clearly support that leucine inhibited a non-lysosomal and Ca^{2+}-independent proteolytic process in ageing (Fig. 4).

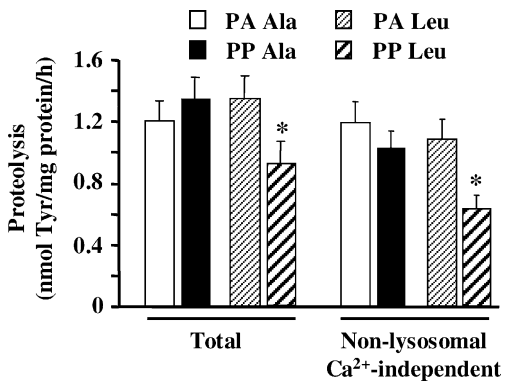

Figure 4. Total and non-lysosomal Ca^{2+}-independent proteolysis in 22-month-old rats chronically fed an alanine- or leucine-supplemented diet
Rats from Experiment 2 received a diet supplemented with either alanine or leucine for 10 days. The day of the experiment they were overnight starved (PA Ala or PA Leu) or fed during 1 h either diet (PP Ala or PP Leu). Rates of total proteolysis and non-lysosomal Ca^{2+}-independent proteolysis were measured in incubated epitrochlearis muscles as described in Methods. Values are means ± S.E.M. (vertical bars) for 9–10 animals and are expressed in nmol of Tyr (mg protein)$^{-1}$ h^{-1}. *$P < 0.05$ *versus* PA Leu as assessed by ANOVA.

Discussion

These findings indicate that the PP inhibition of skeletal muscle ubiquitin–proteasome-dependent proteolysis is defective in 22-month-old animals and can be corrected by a dietary leucine supplementation. Rates

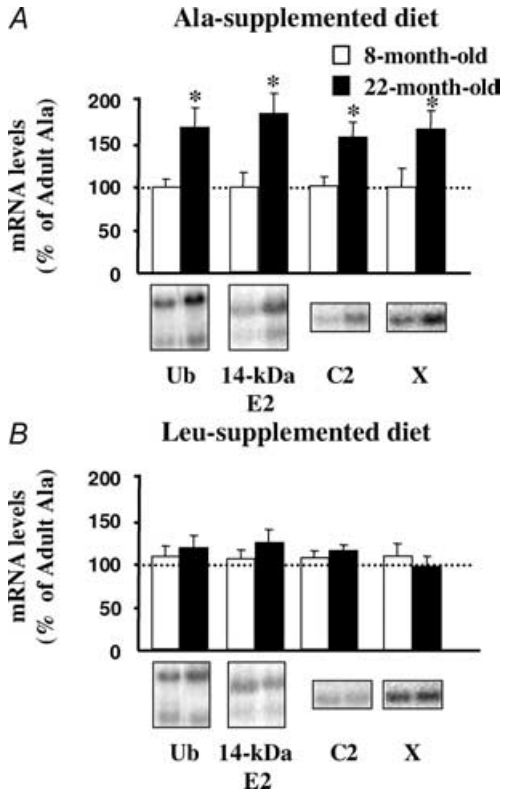

Figure 5. mRNA levels for genes encoding components of the Ub–proteasome pathway in 8- and 22-month-old rats chronically fed an alanine- or leucine-supplemented diet
Eight- and 22-month-old rats from Experiment 2 received for 10 days a diet supplemented with either alanine (*A*) or leucine (*B*). The day of the experiment animals were overnight starved, extensor digitorum longus muscles were harvested, and Northern blots for Ub, the 14-kDa E2, and subunits C2 and X of the 20S proteasome were performed as described in Methods. Hybridization signals were quantified and normalized using the corresponding 18S rRNA signals to correct for uneven unloading. Both transcripts for Ub and the lower transcript 14-kDa E2 were quantified. Values are means ± S.E.M. (vertical bars) for $n = 5$–7 rats, and are expressed as percentage of 8-month-old rats fed the Ala-supplemented diet. Representative Northern blots are also shown. *$P < 0.05$ *versus* 8-month-old rats by the unpaired Student's *t* test.

of proteasome-dependent proteolysis were measured *in vitro* as there is no alternative technique for such measurements *in vivo*. Although such measurements are not quantitative they usually reflect qualitative changes observed *in vivo* in a number of catabolic or anabolic conditions (for example see Kee *et al.* 2003). In large 8- and 22-month-old rats *in vitro* rates of proteolysis can only be measured in a thin muscle (i.e. the epitrochlearis) to avoid the development of hypoxic central cores (Maltin & Harris, 1985). Proteasome activities and ubiquitination rates were measured in the EDL in Experiment 1, as well as mRNA levels in Experiment 2. Both the epitrochlearis and the EDL comprise mostly type II fibres. We have previously reported comparable measurements for these and other type II muscles (i.e. the tibialis anterior and the gastrocnemius) for various parameters of ubiquitin–proteasome-dependent proteolysis (Combaret *et al.* 2004). However, we used 22-month-old rats that do not exhibit atrophy of all studied muscles. The lack of atrophy of the EDL muscle in 22-month-old animals is consistent with previous observations in Wistar rats. A reduction in the mass of this muscle was only detected at 25 months of age (Mosoni *et al.* 2004). Since defects in proteasome-dependent proteolysis did not correlate with a high degree of atrophy of the EDL (or epithrochlearis) muscle, one may hypothesize that the defects reported here in 22-month-old animals were early events that will lead to subsequent muscle atrophy. In addition, mortality increases dramatically (by about 50%) between 22 and 25 months of age in this strain of rats. Thus, we used 22-month-old rats to avoid the selection of survivors that may not reflect an average aged population.

Eight-month-old rats but not 22-month-old animals exhibit decreased *in vitro* proteasome-dependent proteolysis in the postprandial state

Previous studies have detected abnormalities in the regulation of muscle proteolysis in aged muscles (Volpi *et al.* 2001; Arnal *et al.* 2002). Since the Ub–proteasome-dependent proteolytic pathway plays a major role in skeletal muscle (Jagoe & Goldberg, 2001; Hasselgren & Fischer, 2001; Attaix *et al.* 2003), we investigated whether this process was down-regulated in the PP state in 8- and 22-month-old rats. We show that *in vitro* proteasome-dependent proteolysis was down-regulated in the PP state in 8-month-old rats but not in 22-month-old animals (Fig. 1). These findings clearly support the lack of inhibition of overall muscle proteolysis in the PP state previously reported in old rats (Arnal *et al.* 2002), and further show that in standard nutritional conditions the skeletal muscle Ub–proteasome system from old rats was insensitive to meal-induced anabolic stimuli.

The lack of regulation of the ubiquitin–proteasome system in ageing may impair skeletal muscle protein deposition

The lack of responsiveness of the Ub–proteasome system that prevailed in the PP state is not unique in old muscles. Indeed, the Ub–proteasome pathway is not activated in muscles from old rats treated *in vivo* with the synthetic glucocorticoid analogue dexamethasone (Dardevet *et al.* 1995). By contrast, glucocorticoids stimulate muscle Ub–proteasome-dependent proteolysis in adult rats by various mechanisms (Combaret *et al.* 2004). Thus, this major proteolytic machinery became insensitive to both anabolic and catabolic stimuli in ageing. This may have negative consequences on muscle protein deposition, because the efficiency of this process is greatly enhanced with simultaneous changes, even of limited amplitude, in rates of both protein synthesis and breakdown (Waterlow *et al.* 1978). Old rats are only able to slightly increase muscle protein synthesis in response to feeding (Mosoni *et al.* 1995). Thus, our observations may contribute to the explanation of why muscle recovery is strongly impaired in ageing following negative nitrogen balance conditions (e.g. catabolic treatments (Dardevet *et al.* 1995) or starvation (Mosoni *et al.* 1999)). Moreover, the incidence of pathologies, which results in such catabolic conditions (Mitch & Goldberg, 1996; Attaix *et al.* 2003), increases with ageing. Thus, the inability of old animals to alter muscle rates of proteolysis may also contribute to the explanation of the progressive establishment of sarcopenia. Finally, the Ub–proteasome pathway degrades major contractile proteins such as actin and type II myosins (Mitch & Goldberg, 1996; Jagoe & Goldberg, 2001; Hasselgren & Fischer, 2001; Attaix *et al.* 2003), and protein synthesis of myosin heavy chain decreased in ageing (Greenlund & Nair, 2003). Overall, this should impair the ability of the organism to maintain the pool of this very abundant contractile protein in ageing.

The postprandial inhibition of *in vitro* skeletal muscle proteolysis in 8-month-old rats reflects decreased proteasome activities

In 8-month-old rats the decreased proteasome peptidase activities in the PP state (Fig. 2) are consistent with the inhibition of *in vitro* proteasome-dependent rates of proteolysis (Fig. 1). By contrast, muscle proteasome activities slightly increased following meal ingestion in the 22-month-old rats (Fig. 2). These data were repeatedly obtained in two different experiments and were confirmed by measuring proteasome activities using another technique (i.e. partially purified proteasomes on a glycerol gradient (Tilignac *et al.* 2002; Combaret *et al.* 2004; data not shown)). However, rates of ubiquitination

also tended to decrease ($P < 0.06$) in the PP state in the 22-month-old animals (Fig. 3). Overall, the opposite regulation of rates of ubiquitination and of proteasome activities in muscles from 22-month-old animals is presumably responsible for the lack of significant variation in *in vitro* proteasome-dependent proteolysis following meal ingestion (Fig. 1). Alternatively, the increased measured proteasome peptidase activities in the PP state in 22-month-old rats were detected with exogenous fluorogenic substrates. We cannot rule out that these measurements did not reflect proteasome-dependent proteolysis of the actual endogenous muscle substrates.

Ageing decreases muscle proteasome activities in the postabsorptive state

A strong decrease in proteasome activities characterized ageing in the PA state (Fig. 2). Previous studies in ageing reported no change in activity (Radak *et al.* 2002*a*) or decreased activity in either all catalytic sites (Bardag-Gorce *et al.* 1999; Husom *et al.* 2004) or limited to the trypsin-like activity (Radak *et al.* 2002*b*). These discrepancies are not unexpected since proteasome activities are largely influenced by the nutritional status of the animals (Fig. 2), which was not indicated in all these studies. Our findings further show that changes in the rate of ubiquitination in ageing are unlikely to play a major role in age-related disturbances of the Ub–proteasome system (Fig. 3). Taken together, our data support the down-regulation of the pathway in aged muscle as already reported in a variety of tissues (Carney *et al.* 1991; Keller *et al.* 2000; Bulteau *et al.* 2002; Husom *et al.* 2004).

Leucine fully restored the defective postprandial inhibition of proteasome-dependent proteolysis in 22-month-old rats

The leucine-supplemented diet fully restored the defective postprandial inhibition of *in vitro* proteasome-dependent proteolysis in 22-month-old rats (Fig. 1), by normalizing proteasomal activities (Fig. 2) and by decreasing rates of ubiquitination (Fig. 3). These effects are presumably due to the increase in leucine availability since plasma leucine increased twofold in rats receiving the leucine-supplemented meal without significant modification in the levels of other amino acids or insulin (Dardevet *et al.* 2002; Rieu *et al.* 2003). In similar *in vitro* experiments, leucine stimulated protein synthesis (Dardevet *et al.* 2000). By contrast, incubating muscles with 200–600 μM leucine had no effect on proteolysis (data not shown). Furthermore, Mitch & Clark (1984) showed that the inhibitory effect of leucine on muscle proteolysis requires transamination. Taken together these observations suggested that the inhibitory effect of leucine on *in vitro* proteasome-dependent proteolysis in this study is likely to be indirect. Leucine enhances muscle protein synthesis by stimulating the mammalian target of the rapamycin pathway (mTOR), as well as the 70-kDa ribosomal protein S6 kinase activity, and by enhancing eIF4E-binding protein phosphorylation and the association of eukaryotic initiation factor eIF4E with eIF4G, both *in vitro* and *in vivo* (Dardevet *et al.* 2000; Anthony *et al.* 2000*a*,*b*; Greiwe *et al.* 2001). mTOR is the target of Akt, and Akt activation also blocks the up-regulation of atrogin-1/MAFbx (Lee *et al.* 2004), a muscle-specific E3 that is critical for enhanced Ub–proteasome-dependent proteolysis (Bodine *et al.* 2001; Gomes *et al.* 2001). Thus, one may hypothesize that leucine simultaneously stimulated muscle protein synthesis and inhibited the Ub–proteasome pathway by acting on Akt.

Chronic administration of a leucine-supplemented diet has long-lasting effects on the Ub–proteasome system

The elevated mRNA levels for components of the Ub–proteasome pathway in 22-month-old rats fed the control alanine-supplemented diet (Fig. 5*A*) are in accordance with observations in aged human muscle (Welle *et al.* 2003). Furthermore, the increased mRNA levels for the proteasome subunit C2 observed in ageing (Fig. 5*A*) also agrees with an increased protein content for this particular subunit in muscle tissue from old rats (Husom *et al.* 2004). Thus, proteasome activities decreased with ageing in the PA state (Fig. 2) although there was enhanced expression of proteasome subunits (Fig. 5*A*). It should be pointed out that an increased expression (Husom *et al.* 2004 and this experiment) or protein content (Husom *et al.* 2004) of 20S proteasome subunits does not imply high rates of proteasome-dependent proteolysis in ageing. Indeed, the content of the 19S complex is dramatically reduced in the aged muscle and seems inadequate for complete activation of the 20S proteasome catalytic properties (Husom *et al.* 2004; Ferrington *et al.* 2005).

Supplementation of the diet with leucine for 10 days completely suppressed the increased muscle mRNA levels for Ub, the 14-kDa E2, and C2 or X proteasome subunits in 22-month-old rats (Fig. 5*B*). Although such measurements may not necessarily correlate with rates of proteolysis, they strongly suggest that chronic administration of a leucine-supplemented diet has long-lasting effects on the Ub–proteasome system *in vivo*. Furthermore, they further support the observation that leucine inhibited a non-lysosomal and Ca^{2+}-independent proteolytic process in the incubated muscles from 22-month-old rats (Fig. 4).

Since chronic leucine administration has also long-lasting effects on the restoration of enhanced muscle protein synthesis in the PP state in ageing (Rieu *et al.* 2003), our observations strongly suggest that leucine may contribute to the improvement of protein balance in sarcopenia. This is further supported by recent observations indicating that a leucine-supplemented diet stimulated protein synthesis and inhibited the activation of the ubiquitin–proteasome system in the muscles from cancer animals (Ventrucci *et al.* 2004).

Conclusion

The present findings provide evidence for decreased *in vitro* proteasome-dependent proteolysis in the aged muscle. We also demonstrate that the defect in PP anabolism observed in ageing results from a lack of inhibition of Ub–proteasome-dependent proteolysis that can be fully restored by leucine supplementation in the diet. Preventing sarcopenia is a major socioeconomic and public health issue. Our observations suggest that very simple nutritional supports (i.e. leucine supplementation or leucine-rich dietary components) may help the elderly to preserve muscle mass.

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