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RESEARCH ARTICLES

Lysosomal and proteasome-dependent proteolysis are differentially regulated by insulin and/or amino acids following feeding in young, mature and old rats☆

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Abstract

Skeletal muscle proteolysis is inhibited by oral feeding in the young and mature but not in the elderly. However, the proteolytic pathway (s) responsible for the decreased muscle proteolysis in the postprandial (PP) state is (are) unknown in the young. Moreover, muscle proteolysis is inhibited by both insulin (INS) and amino acids (AA) *in vitro*, but their respective roles on specific proteolytic pathways *in vivo* remain to be elucidated. The aim of this study was to investigate the respective role of INS and AA on the inhibition of proteolytic pathways in the PP state in skeletal muscles from young, mature and old rats. Rats were fed over 1 h either a 25% (AA+) or a 0% (AA−) amino acid/protein meal. In each nutritional condition, PP insulin secretion was maintained (AA+/INS+ and AA−/INS+) or blocked (AA+/INS− and AA−/INS−) with diazoxide injections. We report that the PP inhibition of proteolysis in young rats was mediated by the increased INS secretion and resulted from a down-regulation of both lysosomal and Ca^{2+}-dependent proteolysis. Moreover, our data showed that proteasome activities are inhibited by either INS or AA in mature rats, whereas they become selectively insensitive to AA in old rats. In conclusion, the present work provides direct evidence that the lack of PP regulation of proteasome-dependent proteolysis in old rats resulted from a selective resistance to AA.

Keywords: Aging; Insulin; Amino acids; Proteasome-dependent proteolysis; Lysosomal proteolysis

1. Introduction

Amino acids (AA) and insulin (INS) play a major role in promoting postprandial (PP) protein anabolism [1–4]. *In vitro* studies have shown that INS and AA can independently regulate skeletal muscle protein synthesis and degradation [5–7]. However, the concomitant changes in INS and AA plasma concentrations that usually prevail following meal ingestion complicate *in vivo* studies. We and others suggested that (i) AA are essential in PP stimulation of protein synthesis in rodents and humans [1,3,4,8], (ii) INS had a permissive effect on synthesis [9,10] and (iii) both INS and AA were required to stimulate skeletal muscle protein synthesis in response to feeding in young and mature rats [1,8], whereas the effect of dietary AA was blunted in old animals [3].

In contrast, the regulation of muscle proteolysis by AA or INS *in vivo* has been less studied. Both INS and AA were also required for PP inhibition of skeletal muscle proteolysis after feeding [1,8]. Multiple proteolytic pathways, including the lysosomal, Ca^{2+}-dependent and Ub (ubiquitin)–proteasome-dependent processes and other proteases (e.g., caspases and matrix metalloproteases), are responsible for skeletal muscle proteolysis. Previous work from our laboratory showed that the induction of proteolysis induced by AA deprivation in C2C12 myotubes is mainly mediated by the lysosomal system [11,12]. In this process, the increase in protein substrate sequestration involved LC3/Apg8 lipidation [12]. In addition, Mizushima et al. [13] showed

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that macroautophagy is induced by starvation in skeletal muscle using transgenic mice expressing LC3 fused to green fluorescent protein. However, to the best of our knowledge, the respective role of INS and AA on lysosomal proteolysis is unknown in anabolic situations. Starvation/refeeding experiments showed that the ubiquitin–proteasome system, which plays a major role in the breakdown of muscle proteins, is also regulated by AA supply. However, these data are difficult to interpret because fasted animals exhibit reduced INS circulating levels, which regulate the expression of components of the ubiquitin system [14–16]. In AA perfused and refed starved rats, there was no change in proteasome-dependent proteolysis although circulating free AA levels were normalized compared with control rats. Finally, an inhibition of proteasome-dependent proteolysis following feeding prevails in mature rats but is defective in old animals. This defect is restored by leucine supplementation in the diet [17]. Thus, although recent data suggest that physiologic concentrations of some AA may down-regulate peptidase activities of purified skeletal muscle proteasomes [17], further experiments are clearly needed to demonstrate whether AA, independently of INS, may regulate this pathway *in vivo*.

In the present study, we investigated (1) the proteolytic pathways responsible for the inhibition of proteolysis following feeding in young rats and (2) the respective role of INS and AA in the regulation of proteasome-dependent proteolysis in the PP state in young, mature and old animals.

2. Materials and methods

2.1. Animals and experimental design

The present study was performed in accordance with the current legislation on animal experimentation in France. Experiments were performed in young, mature and old male Wistar rats. Animals were housed individually under controlled environmental conditions (temperature 22°C; 12-h dark period starting at 0800 hours), fed *ad libitum* a 15% (young rats; Table 1) or a 18.5% casein diet (mature and old rats [3]) and had free access to water during the adaptation period. Animals were randomly allocated into groups and overnight starved the day before the experimentation. On the day of the experiment, rats received the appropriate diet over 1 h and were sacrificed under anaesthesia 2 h after the end of the feeding period.

After 1 week of adaptation, young rats (6 weeks old and weighing approximately 130 g) were separated into two (Experiment 1) or four groups (Experiment 2) of 10 animals. In Experiment 1, half of the rats were fed a 25 % AA-protein diet over 1 h (PP state; Table 1) after a 16-h fasting, whereas the other half stayed in the post-absorptive (PA) state. In Experiment 2, all rats were studied in the PP state and were fed either a 25% AA-protein diet or a 0% AA-protein diet over 1 h. Food intake was not significantly different among rats over this 1-h period. After feeding, one-half of the animals were subjected to two intraperitoneal injections of either vehicle or diazoxide to suppress PP insulin secretion. The first diazoxide injection was performed at the end of the meal (25 mg/100 g body weight in 0.05 mol/L NaOH) (hyperstat diazoxide, Schering-Plough, France) and the second one 60 min later (18 mg/100 g body weight) as described by Sinaud et al. [8]. In Experiment 3, mature and old rats were subjected to a similar but adapted protocol in terms of diet composition, diazoxide dose and timing. The protocol is described in Prod'Homme et al. [3]. Briefly, mature (8-month-old) and old (22-month-old) rats were acclimated to their surroundings for 1 month; meanwhile, animals were fed between 1000 and 1630 hours *ad libitum* with an 18.5% casein diet. In the 25% AA-protein diet, AA originated from both casein (10% for young rats and 5% for mature or old animals) and a specific AA mixture (15% for young rats and 20% for mature or old animals). A combination of protein and free AA was used to obtain a similar rapid PP increase in plasma AA in diazoxide-treated and control rats.

Table 1
Composition of the diet and experimental meals in Experiment 1

	Casein diet[a]	25 % AA/protein meal	0% AA/protein meal
Casein	15	10	–
Cystine	0.2	–	–
L-Amino acid mixture[b]	–	30	–
Peanut oil	2.4	2.4	4
Colza oil	3	3	5
Sunflower oil	0.6	0.6	1
Saccharose	15	25	25
Agar–agar	3	–	10
Wheat starch	49.8	34	55
Supplements			
Multivitamin mixture	1	–	–
Multimineral–oligoelement mixture	8	–	–
Energy [kcal (100 g dry matter)$^{-1}$]	417	453	492

Composition of the diets is expressed in % of dry matter. They were prepared according to the *Nutrient Requirements of Laboratory Animals* (4th Edition, 1995, National Academy, Washington DC). The two experimental diets were isoenergetic.

[a] Casein diet was from Unité de Préparation d'Aliments Expérimentaux, INRA Jouy-en-Josas, France.

[b] Composition of the L-amino acid mixture was as follows (in grams per 100 grams of mixture): aspartic acid, 6.84; asparagine, 4.58; threonine, 5.29; serine, 4.90; glutamic acid, 10.97; glutamine, 5.87; proline, 4.13; glycine, 2.00; alanine, 4.65; valine, 4.84; cystine, 2.90; methionine, 2.00; isoleucine, 5.03; leucine, 12.32; tyrosine, 3.48; phenylalanine, 3.61; lysine, 9.74; histidine, 1.87; arginine, 2.84; and tryptophan, 2.13.

2.2. Rates of protein breakdown

Muscle protein breakdown was measured exactly as described previously [18] in skeletal muscles from young rats. Extensor digitorum longus (EDL) muscles were carefully dissected and incubated at approximately resting length by pinning their tendons on plastic supports. All muscles were preincubated at 37°C in a Krebs–Henseleit buffer

(120 mmol/L NaCl, 4.8 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, pH 7.4) supplemented with 5 mmol/L Hepes, 5 mmol/L glucose and 0.1% BSA. After 30 min of preincubation, muscles were transferred to a fresh medium of identical composition, saturated with an O_2/CO_2 (19/1) gas mixture and incubated further for 1 h at 37°C. Rates of protein breakdown were measured by following the rates of tyrosine release into the medium in the presence of 0.5 mmol/L cycloheximide to block protein synthesis [19]. The contribution of lysosomal and Ca^{2+}-dependent proteases and of proteasome to the rates of overall proteolysis was determined as described [19]. When measuring proteasome-dependent proteolysis, skeletal muscles were incubated in the presence of lysosomal and Ca^{2+}-dependent protease inhibitors because the proteasome inhibitor MG132 also inhibits cysteine proteases (i.e., cathepsin B, H and L, and calpains) [19]. Protein degradation was expressed in nanomoles of tyrosine released into the medium per milligram of protein per hour. Muscle protein content was measured according to the bicinchoninic acid procedure.

2.3. Chymotrypsin-like activity of the proteasome

Gastrocnemius muscles from young, mature and old rats (n=5 animals per group) were pooled and homogenized in an ice-cold buffer (pH 7.5) containing 50 mmol/L Tris, 250 mmol/L sucrose, 10 mmol/L ATP, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT) and protease inhibitors (10 μg/ml of antipain, aprotinin, leupeptin and pepstatin A, and 20 mmol/L phenylmethylsufonylfluoride). Proteasomes were isolated by three sequential centrifugations as previously described [17]. The final pellet was resuspended in a buffer containing 50 mmol/L Tris (pH 7.5), 5 mmol/L $MgCl_2$ and 20% glycerol. The protein content of the proteasome preparation was determined according to Lowry et al. [20]. The chymotrypsin-like activity of proteasome was determined by measuring the hydrolysis of the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (suc-LLVY-AMC) (Sigma). To measure peptidase activity, 10 μl of the proteasome extract was added to 40 μl of medium containing 50 mmol/L Tris (pH 8.0), 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 2 U apyrase and 300 mmol/L of suc-LLVY-AMC. The peptidase activity was determined by measuring the accumulation of the fluorogenic cleavage product (amido-4-methylcoumarin, AMC) using the FLX800 fluorimeter (Bio-Tek, Saint Quentin, France) with 380- and 440-nm excitation and emission wavelengths, respectively. The difference between arbitrary fluorescence units recorded with or without 40 μmol/L 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 after hydrolysis of the substrate was analysed by linear regression to calculate peptidase activities, i.e., the slopes of best fit of accumulated AMC vs. time. Values were expressed in relative fluorescence unit per microgram of protein per minute.

2.4. Plasma INS and AA measurements

Plasma INS concentrations were analyzed using a commercial radioimmunoassay kit (Bio-Rad, France). Plasma free AA were determined by ion-exchange chromatography after protein precipitation: 500 μl of plasma was added to 125 μl of a sulfosalicylic acid solution (1 mol/L in ethanol with 0.5 mol/L thioglycol) previously evaporated to dryness. Samples were incubated on ice for 1 h and then centrifuged at 3500×g at 4°C for 1 h. An aliquot (250 μl) of the supernatant was combined with 125 μl of 0.1 mol/L lithium acetate buffer (pH 2.2). Amino acid concentrations were determined on extracts by an AA analyzer (HPLC system; Bio-Tek Instruments) (Table 2).

2.5. Statistical methods

Values are given as means±S.E.M. The effect of meal ingestion on proteolytic pathways in Experiment 1 was assessed using the Student's t test. The effect of type of meal, INS secretion and their interactions in Experiment 2 were analyzed using a two-way ANOVA. When significant overall effect was detected, differences among individual means were assessed with the PLSD Fisher's test. In Experiment 3, standard errors for slopes (e.g., proteasome activity) were computed as described by Sokal and Rohlf [21]. Chymotrypsin-like activities were compared according to Snedecor and Cochran [22]. This statistical procedure involves first a comparison of residual variances by Bartlett's test. Assuming homogeneity of residual variances, slopes were then compared by the F test. Differences among means were considered significant when $P \leq .05$. The Statview

Table 2
Plasma branched-chain AA concentrations in young rats

	25% AA/protein		0% AA/protein		Two-way ANOVA, significant effects		
	INS+/AA+	INS−/AA+	INA+/AA−	INS−/AA−	M	I	MI
Leucine	427±92^{AC}	551±121^{A}	104±15^{B}	236±19^{BC}	<0.005	ns	ns
Isoleucine	182±40^{AC}	213±39^{A}	58±6^{B}	133±12^{C}	<0.02	ns	ns
Valine	429±76^{A}	378±77^{A}	111±13^{B}	211±16^{B}	<0.001	ns	ns

Plasma branched-chain AA concentrations (nanomoles per gram of plasma) were measured in the four groups: INS+/AA+, fed a 25% AA/protein meal in the presence of INS secretion; INS−/AA+, fed a 25% AA/protein meal in the absence of INS secretion; INS+/AA−, fed a 0% AA/protein meal in the presence of INS secretion; INS−/AA−, fed a 0% AA/protein meal in the absence of INS secretion. Values are means±S.E.M. for four determinations per group. Two-way ANOVA was performed to discriminate among effect of type of meal (M), insulin (I) and their interaction (MI) on AA concentrations; ns, not significant. PLSD Fisher's test was performed a posteriori and values within a line with same letters are not significantly different.

program (version 5.0, Abacus Concepts, Berkley, CA, USA) was used for statistical analyses.

3. Results

3.1. Proteolytic pathways inhibited following feeding in young rats (Experiment 1)

Fig. 1A shows that total proteolysis was reduced by 32% in EDL muscles following meal ingestion. Only inhibitors of cathepsins and calpains blocked this effect, suggesting that the lysosomal and the Ca^{2+}-dependent proteolytic pathways are responsible for the inhibition of proteolysis following feeding in young rats. Indeed, Fig. 1A shows that lysosomal and Ca^{2+}-dependent proteolysis decreased by 67% following feeding, whereas proteasome-dependent proteolysis remained unchanged.

Fig. 1B shows that both INS and branched-chain AA levels increased by 400% and 156%, respectively, 2 h after the end of the feeding period. Since both parameters mediate protein metabolism changes, we studied the respective role of INS and AA in the inhibition of total and lysosomal and Ca^{2+}-dependent proteolysis.

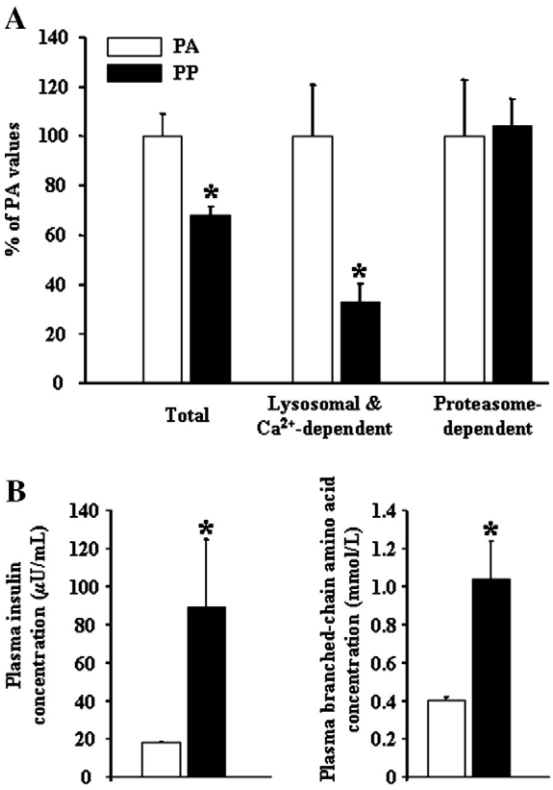

Fig. 1. Regulation of skeletal muscle proteolytic pathways in young rats in the PA state or following feeding with a 25% AA-protein meal (Experiment 1). EDL muscles were excised and incubated *in vitro* in the absence or presence of specific protease inhibitors, as described in Materials and Methods. (A) Rates of total, lysosomal and Ca^{2+}-dependent and proteasome-dependent proteolysis. Values are expressed in % of PA values. (B) Plasma INS and branched-chain AA concentrations. Plasma INS concentrations were determined by RIA with an INS assay kit. Plasma branched-chain AA levels were determined by ion-exchange chromatography as described in Materials and Methods. In both panels, values are means±S.E.M. (vertical bars, n=10). ∗Significant changes were determined using the unpaired Student's t test (P<05 vs. the PA group).

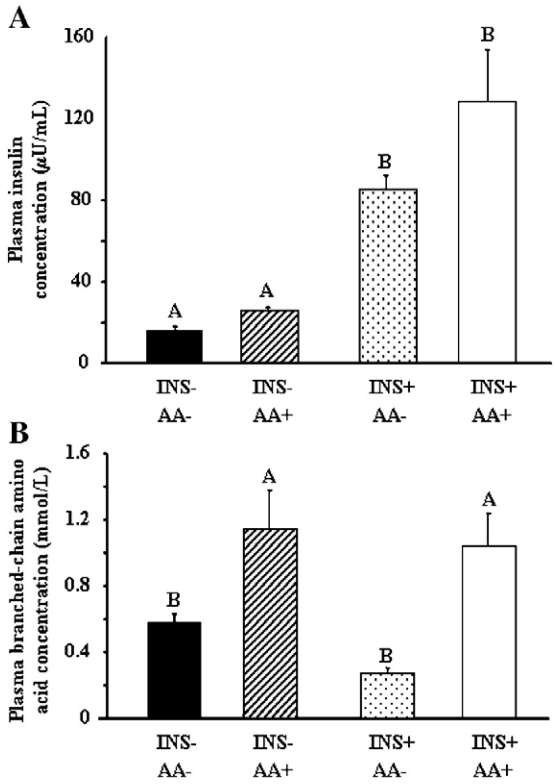

Fig. 2. Plasma INS and branched-chain AA concentrations in young rats fed a 25% (AA+) or 0% (AA−) amino acid/protein meal without (INS+) or with (INS−) diazoxide treatment (Experiment 2). (A) Plasma INS concentrations were determined by RIA with an INS assay kit. (B) Plasma branched-chain AA levels were determined by ion-exchange chromatography as described in Materials and Methods. In both panels, values are means±S.E.M. (vertical bars, n=10). Columns with different letters are significantly different (P<05).

3.2. Regulation of skeletal muscle proteolysis by INS and AA in young rats (Experiment 2)

Rats were studied in the PP state to discriminate the impact of INS and AA on the inhibition of proteolysis in skeletal muscles. Amino acid levels following meal ingestion were manipulated using either a 25 % AA-protein or a 0% AA-protein diets. The increased INS plasma levels after feeding were blunted in half of the animals using diazoxide, which blocks INS secretion. This protocol was designed to obtain animals where (i) neither INS nor AA levels were elevated (INS−/AA−), (ii) only plasma AA levels increased (INS−/AA+), (iii) only plasma INS increased (INS+/AA−) or (iv) both INS and AA levels were elevated (INS+/AA+) (Fig. 2A and B).

Taking the INS−/AA− group as a reference, Fig. 3A shows that the elevation of AA levels alone (INS−/AA+ group) did not reduce total proteolysis at all. By contrast, the

rise in plasma INS alone (INS+/AA− group) decreased proteolysis by 27% as observed in the INS+/AA+ group. Thus, these data show that INS was required and compulsory for decreasing total proteolysis in the PP state in young rats.

Fig. 3B shows that the selective elevation of AA (INS−/AA+ group) or INS (INS+/AA− group) levels partially reduced lysosomal and Ca^{2+}-dependent proteolysis by 32% (NS) compared with the INS−/AA− group. However, these proteolytic rates were not statistically different from those measured in the INS+/AA+ group. By contrast, the inhibition was exacerbated and reached 59% ($P<.05$) when both INS and AA were elevated (INS+/AA+ group) compared with the INS−/AA− group, suggesting that both factors were required for the complete inhibition of these proteolytic pathways.

3.3. Regulation of skeletal muscle proteasome activities by INS and AA following feeding during aging (Experiment 3)

Our previous data showed that the defect in PP anabolism observed in aging results from a lack of inhibition of Ub–proteasome-dependent proteolysis, the peptidase activities of the proteasome becoming insensitive to meal ingestion between the ages of 8 and 22 months [17]. However, the mechanisms underlying the decreased sensitivity in aging of this particular proteolytic pathway are totally unclear. Thus, we investigated the regulation of 20S proteasome peptidase activities by INS and AA in gastrocnemius muscles from young, mature and old rats.

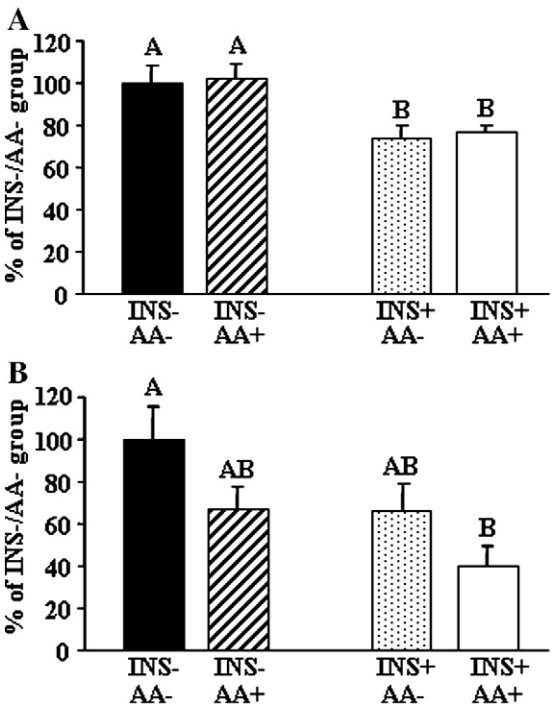

Fig. 3. Total and lysosomal and Ca^{2+}-dependent proteolysis in skeletal muscles from young rats fed a 25% (AA+) or 0% (AA−) amino acid/protein meal without (INS+) or with (INS−) diazoxide treatment (Experiment 2). EDL muscles were excised and incubated *in vitro* in the absence or presence of inhibitors of lysosomal and Ca^{2+}-dependent proteases, as described in Materials and Methods. (A) Total proteolysis; (B) lysosomal and Ca^{2+}-dependent proteolysis. Values are expressed in % of INS−/AA− group and are means±S.E.M. (vertical bars, $n=10$). Columns with different letters are significantly different ($P<.05$).

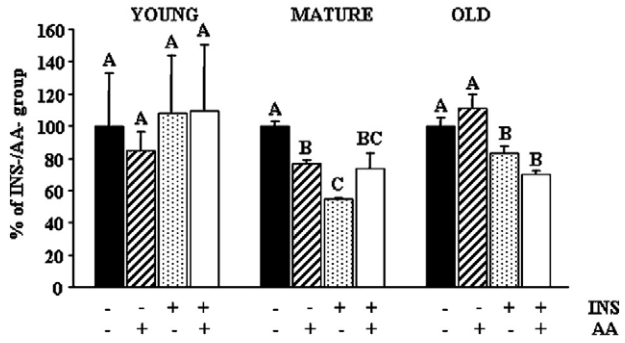

Fig. 4. Chymotrypsin-like activity of the proteasome in skeletal muscles from young, mature and old rats fed a 25% (AA+) or 0% (AA−) amino acid/protein meal without (INS+) or with (INS−) diazoxide treatment (Experiment 3). Data represent the slopes of best fit of arbitrary fluorescence units released from Suc-LLVY-AMC fluorescent substrate vs. time. Values are expressed in % of INS−/AA− group and are means±S.E.M. (vertical bars). Columns with different letters are significantly different ($P<.05$).

According to the absence of proteasome-dependent proteolysis inhibition following feeding in young rats (Fig. 1A), the chymotrypsin-like activity of the 20S proteasome was not regulated by either INS or AA (Fig. 4). In mature rats, the chymotrypsin-like activity of the 20S proteasome decreased in the INS−/AA+ (−23 %) and INS+/AA− (−46 %) groups compared with the INS−/AA− group. When both INS and AA levels increased (INS+/AA+), the inhibition was not statistically different from that observed in the INS+/AA− and INS−/AA+ groups (Fig. 4), suggesting a similar role for INS and AA in the regulation of proteasomal activity compared to the INS−/AA− group. In old animals, the chymotrypsin-like activity was unchanged between the INS−/AA+ and the INS−/AA− groups. By contrast, increased plasma INS concentration was sufficient to reduce this peptidase activity by 17% or 30% in the INS+/AA− and INS+/AA+ groups, respectively (Fig. 4).

4. Discussion

We report in young rats that (i) meal ingestion decreased skeletal muscle protein breakdown via the selective inhibition of lysosomal and Ca^{2+}-dependent proteolysis and (ii) the increase in both plasma INS and AA levels was required to regulate this proteolytic pathway following feeding. By contrast, we previously demonstrated that the proteasome system was responsible for the inhibition of proteolysis in mature rats following feeding and that this pathway becomes insensitive to meal ingestion during aging [17]. We demonstrated here that, in the PP state, peptidase activities of the proteasome (i) were not regulated in young rats, (ii)

decreased when INS or AA plasma levels increased in mature rats and (iii) were no more sensitive to increased AA plasma levels in old rats.

It is well known that feeding increases protein synthesis and decreases proteolysis in muscles from young rats leading to protein deposition. However, we report here that feeding inhibited selectively lysosomal and Ca^{2+}-dependent proteolysis. This is surprising because skeletal muscle contains few lysosomes and the major lysosomal proteases (i.e., cathepsins B, H, L and D) are not believed to play a major role in overall protein breakdown in muscles incubated under optimal conditions [23,24]. Like cathepsins, calpains are not systematically regulated in various conditions and are not directly responsible for the breakdown of actin and myosins [24–26]. Finally, we previously reported that feeding selectively inhibited the proteasome-dependent proteolytic pathway in muscles from mature rats [17]. Altogether, the present observations suggest that the inhibition of proteolysis following meal ingestion did not target the same protein substrates in growing and mature rats. Thus, the feeding-induced inhibition of the lysosomal and Ca^{2+}-dependent proteolytic systems may not directly control the degradation of myofibrillar proteins in muscles from growing rats. Indeed, Bates and Millward [27] showed that there was a disproportionate increase in myofibrillar protein synthesis during growth. Thus, the stimulation of myofibrillar protein synthesis following meal ingestion in young rats may be sufficient to ensure gain of muscle protein mass, whereas the inhibition of lysosomal and Ca^{2+}-dependent proteolysis may serve to sharply control the degradation of some specific regulatory proteins involved in that process.

In the present study, we showed that INS alone inhibited the rates of total proteolysis and that AA had no additional effect on the skeletal muscles of young rats in the PP state. This is in agreement with previous data from our laboratory [1]. By contrast, inhibition of the lysosomal and Ca^{2+}-dependent proteolytic pathways required both elevated INS and AA levels. In addition, our results showed that the proteasome-dependent system was not regulated by feeding in young rats. This suggests that the decrease in total proteolysis induced by INS alone should involve the down-regulation of other proteases, such as caspases or metalloproteinases. In accordance with the lack of regulation of proteasome-dependent proteolysis, we showed here that the peptidase activities of 20S proteasome were not regulated by increased INS and/or AA levels in young rats in the PP state. This result is closely akin to our previous observations showing that AA starvation stimulates lysosomal proteolysis in myotubes by induction of autophagic complexes [11,28]. We have also demonstrated that INS inhibited proteasome-dependent proteolysis in the EDL muscle from young rats [14]. However, the elevation of INS plasma levels was not comparable in these two distinct experiments. In the study from Larbaud et al. [14], the infusion of INS induced a 60-fold increase in INS plasma levels compared with control animals. In the present work, INS plasma levels increased by fivefold in the PP state compared with the PA state. Altogether, these observations suggest that the proteasome-dependent proteolytic pathway is sensitive to INS, but that there is a threshold of INS induction below which this pathway may not be regulated.

It is well documented that there is an alteration in the proteasome activity with age. We have recently reported a lack of inhibition of the proteasome-dependent proteolysis following feeding in old rats compared with mature animals. This defect is associated with a large decrease in peptidase activities of the proteasome in muscles [17]. Here, we showed that both INS and AA inhibited the chymotrypsin-like activity of the proteasome in skeletal muscles from mature rats. These results suggested that the maintenance of skeletal muscle mass in mature rats is accounted for by both increased protein synthesis and decreased protein breakdown following feeding. Prod'Homme et al. [3] demonstrated that INS is required for the regulation of muscle protein synthesis irrespective of age and that the effect of dietary AA is blunted in old rats. Our data showed that the inhibition of the chymotrypsin-like activity by INS is preserved in old rats, whereas a lack of regulation by AA was detected. This is in good agreement with our previous work, which showed that the defect in proteasome-dependent proteolysis inhibition following feeding was totally restored when rats received a leucine-enriched diet [17]. Altogether, these data showed that the regulation of skeletal muscle proteasome-dependent proteolysis is not resistant to INS but becomes insensitive to AA in old animals.

In conclusion, we report here that the lysosomal, Ca^{2+}-dependent and proteasome-dependent proteolytic pathways are sequentially regulated by AA following feeding during maturation and aging. The study of the respective role of INS and AA in the inhibition of proteolysis in the PP state suggested a threshold of INS induction below which proteasome-dependent proteolysis may not be regulated. Finally, our data provide evidence that the lack of regulation of skeletal muscle proteasome-dependent proteolysis in old rats is mainly mediated by a resistance to AA. Further experiments are clearly needed to improve our understanding of the regulation of skeletal muscle proteolysis during aging using complementary nutritional approaches (i.e., leucine-rich proteins, pulse protein feeding and 'fast' protein concept).

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