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ORIGINAL ARTICLE

Down-regulation of the ubiquitin–proteasome proteolysis system by amino acids and insulin involves the adenosine monophosphate-activated protein kinase and mammalian target of rapamycin pathways in rat hepatocytes

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Abstract The purpose of this work was to examine whether changes in dietary protein levels could elicit differential responses of tissue proteolysis and the pathway involved in this response. In rats fed with a high protein diet (55%) for 14 days, the liver was the main organ where adaptations occurred, characterized by an increased protein pool and a strong, meal-induced inhibition of the protein breakdown rate when compared to the normal protein diet (14%). This was associated with a decrease in the key-proteins involved in expression of the ubiquitin–proteasome and autophagy pathway gene and a reduction in the level of hepatic ubiquitinated protein. In hepatocytes, we demonstrated that the increase in amino acid (AA) levels was sufficient to down-regulate the ubiquitin proteasome pathway, but this inhibition was more potent in the presence of insulin. Interestingly, AICAR, an adenosine monophosphate-activated protein kinase (AMPK) activator, reversed the inhibition of protein ubiquination induced by insulin at high AA concentrations. Rapamycin, an mammalian target of rapamycin (mTOR) inhibitor, reversed the inhibition of protein ubiquination induced by a rise in insulin levels with both high and low AA concentrations. Moreover, in both low and high AA concentrations in the presence of insulin, AICAR decreased the mTOR phosphorylation, and in the presence of both AICAR and rapamycin, AICAR reversed the effects of rapamycin. These results demonstrate that the inhibition of AMPK and the activation of mTOR transduction pathways, are required for the down-regulation of protein ubiquitination in response to high amino acid and insulin concentrations.

Keywords High protein diet · Protein degradation · Liver · AMPK · mTOR

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Introduction

In mammalian tissues, protein degradation involves three main pathways; i.e., the ubiquitin–proteasome, lysosomal, and the Ca^{2+}-dependent systems. The ubiquitin–proteasome system is the principal machinery degrading cytosolic protein and is responsible for a considerable proportion of cellular protein degradation, mainly of short-lived or abnormal proteins (Hamel et al. 2004). Protein ubiquitination is a multiple-step process mediated by the ubiquitin enzymes E1, E2 (14 kDa-E2), and E3 (Attaix et al. 2001). Proteins labeled by ubiquitin are delivered to the proteasome or lysosomes for degradation, depending on the number of ubiquitins attached to the protein and the lysyl residues to which subsequent ubiquitins are attached (Hicke and Dunn 2003; Elsasser and Finley 2005). The lysosomal system (also called autophagy) degrades long-life proteins and organelles, including the mitochondrion and peroxisomes. This system mainly acts through a complex cellular process of macroautophagy that involved cytoplasm sequestration into vesicles and delivery to a degradative lysosome that contains a range of hydrolases. Of these hydrolases,

cathepsin is able to degrade proteins and requires the involvement of autophagy-related genes (Atg) for autophagosome formation (Klionsky and Emr 2000; Levine and Klionsky 2004). The third pathway is the calcium-dependent proteolytic system that involves calpain as the proteinase (Ilian and Forsberg 1992; Goll et al. 2003; Costelli et al. 2005). Calpain may also be required for macroautophagy in mammalian cells (Otani et al. 2004; Demarchi et al. 2006).

Protein degradation systems and proteolysis are sensitive to the nutritional state (Kettelhut et al. 1988). Proteolysis is increased in the postabsorptive state and during fasting and inhibited under feeding and an increase in habitual protein intake induces both more pronounced fasted state increase and more pronounced meal-induced inhibition of proteolysis, respectively (Price et al. 1994; Forslund et al. 1998). High amino acid concentrations and insulin are the main inhibitors of protein degradation, whereas glucagon and low concentrations of amino acids are the principal stimulators (Gelfand and Barrett 1987; Flakoll et al.1989; Mortimore et al. 1989; Kadowaki et al. 1992; Blommaart et al. 1997; Boirie et al. 1997; Balage et al. 2001; Kanazawa et al. 2004; Waterlow 2006; Capel et al. 2008). In muscle, inhibition of the ubiquitin–proteasome pathway appears to be responsible for the postprandial inhibition of proteolysis in mature rats (Combaret et al. 2005), and during fasting there is an increase in ubiquitin expression that is associated with elevated levels of ubiquitinated proteins in parallel with the rate of proteolysis (Medina et al. 1991). These changes were reversed by refeeding and may at least be due to a direct effect of insulin on 14-kDa ubiquitin-conjugating enzyme expression (Wing and Banville 1994). Lysosomal pathways account for approximately only 10–20% of total skeletal muscle proteolysis, under short-term starvation (Mitch and Goldberg 1996; Attaix et al. 1998). Starvation has been shown to induce macroautophagy in the liver, (Mortimore et al. 1983; Mortimore et al. 1989; Bleiberg-Daniel et al. 1994) and to increase the expression of 14-kDa ubiquitin-conjugating enzyme in the liver and kidney (Wing and Banville 1994). Furthermore, in rat hepatocytes, adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) transduction pathways are involved in the control of autophagic proteolysis (Kanazawa et al. 2004; Meley et al. 2006).

The aim of the present study was to further evaluate individual tissue responses to protein feeding which may differ from each other or from the global trend. Indeed, these individual responses were rarely investigated or limited to skeletal muscle for evident physiological interest and despite the importance of other organs such as the liver in protein metabolism (Chevalier et al. 2009). In addition, the AMPK and mTOR transduction pathways have been previously shown to be involved in the control of liver translation in response to a protein intake in the rat (Chotechuang et al. 2009), but whether they also control protein degradation was not addressed. In order to better understand these processes, the present study determined the consequence of high protein feeding on proteolysis and proteolysis pathways in both the fasted and the fed state in the liver, kidney, and muscle of the rat, and also examined whether amino acids, insulin and the AMPK and mTOR transduction pathways are involved in the control of liver protein degradation in response to protein feeding.

Materials and methods

Animals

Male Wistar rats ($n = 74$) were first of all purchased from Harlan (Horst, The Netherlands) and were used according to the guidelines of the French National Animal Care Committee. The rats were placed under a reversed light rhythm (lights from 20:00 to 08:00 hours) and adapted to these experimental conditions for 1 week. They were then randomly allocated to receive either a normal protein (NP) or a high protein (HP) diet (the composition of the diets are detailed on supplemental data). Both diets were iso-energetic (14.89 kJ/g). The rats were accustomed to receiving their food in two meals: an initial, small meal of 6 g dry matter (DM) between 09:00 and 10:00 hours, and a second meal with a free access to food between 14:00 and 18:00 hours. After the first week, the rats had become used to finishing their first meal within 1 h. All animals had free access to water.

Experimental design

Four separate experiments were carried out in accordance with the guidelines of the French Committee for Animal Care and the European Convention on Vertebrate Animals Used for Experimentation.

Experiment 1

Measurement of ex vivo tissue proteolysis ($n = 26$) On day 15, half of the rats in each group were studied in the fasted state (16 h fast) and half in the fed state (2 h after the start of the meal). The animals were anesthetized with an IP injection of sodium pentobarbital (50 mg/kg BW). Hepatocytes were prepared as previously described (Azzout-Marniche et al. 2007). Protein degradation fluxes were measured on hepatocytes (8×10^6), kidney slices, and soleus muscle by determining the release of an indispensable amino acid in an amino acid-free medium in the

presence of a protein synthesis inhibitor (cycloheximide, Sigma–Aldrich, St. Louis, MO, USA) (Tischler et al. 1982). At the end of incubation, cells or tissues were rapidly separated from their incubation medium by spinning in a micro centrifuge (1 s), frozen in liquid nitrogen and stored at −20°C for the assessment of in vitro tissue protein degradation and the analysis of nitrogen and amino acids as described in supplemental data.

Experiment 2

Analysis of the gene expression of key proteolysis enzymes using real-time PCR (n = 24) Rats were studied in the fasted or fed state, as described in experiment 1. After incision of the abdomen, the liver, kidneys and soleus muscles were quickly harvested under sterile conditions, frozen in liquid nitrogen and stored at −70°C until analysis. Total RNA was extracted and reverse transcribed and the cDNA was used to measure the expression of cathepsin D, Atg3, ubiquitin 14 kDa E2 enzyme, C2 subunit of 20S proteasome, m-calpain and 18S. The analysis of gene expression and the primers used are detailed in supplemental data.

Experiment 3

Postprandial time course of protein ubiquitination (n = 24) After an overnight fast, six rats previously adapted to the NP or HP diet were sacrificed in the fasted state and used as controls. The other nine rats receiving each diet were divided into three groups that were sacrificed 30, 60 or 120 min after the beginning of their standardized meal ($n = 3$ per group on each diet). The livers were quickly harvested and frozen in liquid nitrogen and stored at −70°C until Western blot analysis as described in supplemental data.

Experiment 4

Effect of amino acids, insulin, AICAR and rapamycin on protein ubiquitination in rat hepatocytes Prior to the experiments, the rats used for in situ liver perfusion studies were allowed free access to a commercial laboratory chow diet and water for at least 1 week. Hepatocytes were isolated from the livers of fed rats as described in "Experiment 1" and were seeded at a density of 7×10^6 cells/dish in 100-mm Petri dishes. After cell attachment (4 h) and overnight incubation, the medium was replaced a fresh one corresponding to M199 medium salts supplemented with 5.5 mM glucose and the amino acid (AA) concentration found in the portal vein of NP fasted rats, as previously described (Azzout-Marniche et al. 2007). The hepatocytes were then incubated for 60 min in low or high amino acid concentrations (corresponding to the concentrations measured in the portal vein of NP fasted rats or HP fed rats, respectively, as previously described (Azzout-Marniche et al. 2007) with or without insulin (100 nM) (Sigma–Aldrich), AICAR (500 µM) and/or rapamycin (40 nM) (Cell Signaling Technology, Beverly, MA, USA). Each treatment was performed in duplicate. At the end of the incubation, the hepatocytes were lysed and the proteins extracted were subsequently analyzed using Western blot.

Statistics

Data are expressed as mean ± SD. The effects of diet (NP vs. HP) and meal (fasted vs. fed state) or amino acids (low AA vs. high AA) and conditions (with vs. without insulin, AICAR and/or rapamycin) and their interactions were analyzed using two-way ANOVA (SAS 9.1, SAS Institute, Cary, USA). Post hoc Tukey tests for multiple comparisons were performed to enable pair-wise comparisons. Differences were considered to be significant at $P < 0.05$.

Results

Influence of HP feeding on tissue protein contents and tissue protein degradation rates in vivo

The protein content was significantly increased in hepatocytes in HP rats as compared to NP rats whereas no changes were observed regarding hepatocyte mass (Table 1). The kidney mass was slightly higher in rats fed an HP diet when compared with NP rats, but the protein content was similar whatever was the diet (Table 1). In muscles there were no changes to either protein content or mass (Table 1). Tissue breakdown rates, measured from the ex vivo incubation (Fig. 1), showed that the release of valine from incubated hepatocytes was more strongly inhibited by 42% by the meal in HP rats (10.0 ± 2.6 and 5.3 ± 2.7 nmol/gP/h in the fasted and fed states, respectively) than in NP rats (10.9 ± 2.6 and 9.2 ± 2.8 nmol/gP/h in the fasted and fed states, respectively). Kidney breakdown rates were differently affected by meal ingestion in NP and HP rats (diet × meal interaction, $P < 0.05$). In HP rats, the fed state breakdown rate measured in the kidneys only reached 56% of that measured in NP rats, although this difference was not significant. Muscle protein degradation rates were influenced neither by the diet nor by the meal.

Influence of HP feeding on the gene expression of proteins involved in tissue proteolysis and on ubiquitinated proteins in liver

As reported in Fig. 2, the expression of ubiquitin and 14 kDa enzyme in the liver was significantly influenced by

Table 1 Tissue mass and composition in rats fed NP or HP diets for 14 days

	NP		HP		Stat effect[A]
	Fasted	Fed	Fasted	Fed	
Hepatocytes					
Mass (g)	9.3 ± 1.0	10.4 ± 0.8	10.1 ± 1.3	10.3 ± 1.3	NS
Protein[B] (g/100 g tissue)	6.20 ± 1.35[a]	6.57 ± 1.44[a,b]	8.24 ± 1.32[b]	7.62 ± 0.92[a,b]	D
Kidneys					
Mass (g)	2.17 ± 0.39	2.15 ± 0.44	2.43 ± 0.39	2.45 ± 0.45	D
Protein (g/100 g tissue)	10.4 ± 0.9	10.6 ± 1.1	10.4 ± 0.4	10.5 ± 1.5	NS
Soleus muscles					
Mass (g)	0.258 ± 0.024	0.267 ± 0.037	0.272 ± 0.033	0.265 ± 0.034	NS
Protein (g/100 g tissue)	18.9 ± 0.8	19.0 ± 1.0	19.0 ± 0.7	18.4 ± 1.0	NS

Values are mean ± SD, $n = 24$. Means with a different superscript lower case letters within a row are significantly different, $P < 0.05$ (post hoc Tukey tests for multiple comparisons)

NS not significant

[A] Results from two-way ANOVA with *D* diet effect (NP vs. HP), *M* meal effect (fasted vs. fed), *PxM* diet by meal interaction

the diet ($P < 0.05$) and the meal, which translated into a significant inhibition of hepatic ubiquitin expression by the meal in the HP group, whereas no effect was observed in the NP group (Fig. 2). Cathepsin D mRNA was also decreased in HP-fed rats and there was a significant interaction between the diet and the meal. In the kidneys, the only effect was that the expression of Atg3, an E2-like enzyme responsible for autophagosome formation, was significantly influenced by the meal but not the diet. In muscle, inverse variations were observed regarding the expression of ubiquitin and E2 enzymes. mRNA encoding for ubiquitin increased after meal intake whereas E2 mRNA levels decreased. However, the level of protein intake had no effect on either ubiquitin or E2 expression. In order to confirm the effect of the HP diet on the ubiquitin–proteasome system in the liver, we examined whether this decrease in ubiquitin expression was associated with changes to ubiquitinated proteins. For polyubiquitinated proteins, only the bands between 216 and 62 kDa were quantified. No difference was found between the NP and HP groups in the fasted state (Fig. 3). By contrast, ubiquitinated proteins were reduced (less markedly stained) in HP-fed rats at 30, 60, and 120 min when compared with the fasted state, whereas no changes over time appeared in the NP group.

Effect of amino acids and insulin on ubiquitinated proteins in primary rat hepatocytes

We had previously reported that the HP diet was characterized by an increase in portal amino acid levels; while plasma insulin levels were the same as those seen in rats fed an NP diet (Azzout-Marniche et al. 2007). We therefore investigated the respective roles of amino acids and insulin in down-regulation of the ubiquitin–proteasome system in the liver by the HP diet, using primary hepatocyte culture experiments. We observed that a high amino acid concentration reduced the quantity of ubiquitinated protein (less markedly stained) when compared with low amino acid concentrations without insulin (Fig. 4) (amino acid effect $P < 0.0001$ and insulin effect $P < 0.001$). In the presence of insulin, high amino acid levels more markedly reduced ubiquitinated protein levels ($P < 0.05$) (Fig. 4), whereas low amino acid concentration had no significant effect.

Role of AMPK and mTOR transduction pathways in the control of protein ubiquitination in primary rat hepatocytes

In order to investigate the role of AMPK and mTOR in control of the ubiquitin–proteasome systems, hepatocytes were cultivated with or without AICAR (the activator of AMPK) and/or rapamycin (the inhibitor of mTOR). First of all, we checked on the effect of amino acids and insulin on mTOR and AMPK phosphorylation state (P-mTOR and P-AMPK, respectively) and on one of the well known target of mTOR, the S6 protein (P-S6). The results showed a reduction in P-AMPK at a high AA concentration (Fig. 5a), while either high AA levels or insulin increased mTOR and S6 phosphorylation (Fig. 5b, c), which is in line with our previous report (Chotechuang et al. 2009). Secondly, we studied the effect of AICAR and rapamycin on AMPK and mTOR phosphorylation state. As expected, P-AMPK increased in the presence of AICAR (Fig. 5a), and P-mTOR decreased under both low and high amino acid concentrations in the presence of rapamycin (Fig. 5b). This was associated by the decrease in P-S6. Rapamycin

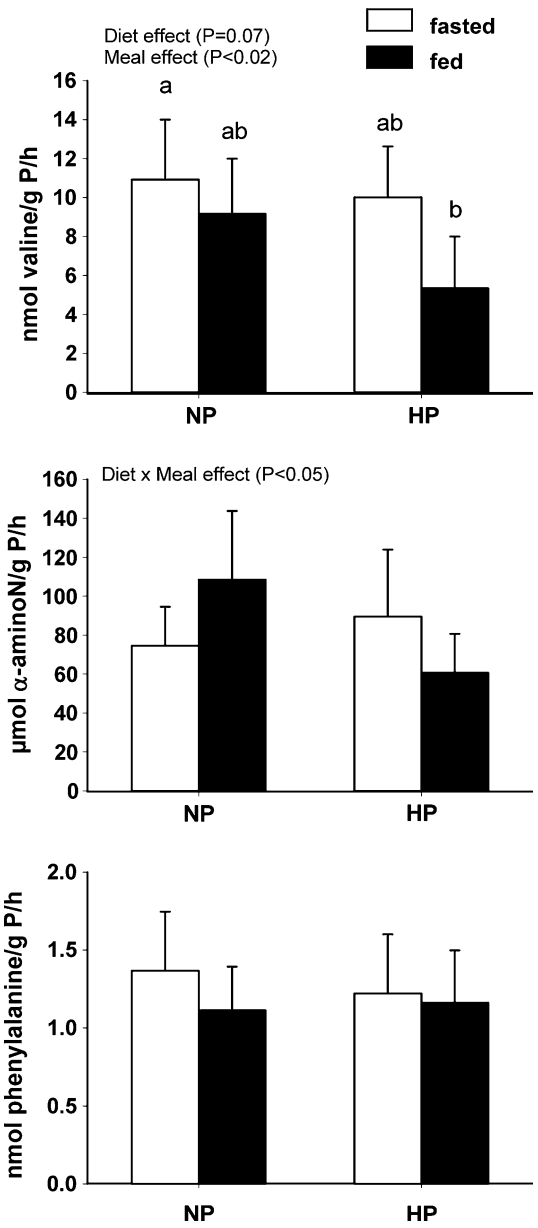

Fig. 1 Protein degradation rates in liver, kidneys, and muscle (soleus). Protein degradation rates were expressed as the amounts of amino acid released by tissue mass in rats adapted to NP or HP diets for 14 days ($n = 26$) and sacrificed in the fasted or fed state. Tissues were incubated in the presence of cycloheximide as described in "Experimental design". Values are mean ± SD. The effects of diet (NP or HP) or meal (fasted or fed) were assessed by two-way ANOVA. *Bars* with *different letters* within a graph are statistically different (post hoc Tukey tests for multiple comparisons, $P < 0.05$)

had no effect on P-AMPK (Fig. 5a) while AICAR decreased P-mTOR and P-S6 under both low and high AA levels in the presence of insulin (Fig. 5b, c). Both AICAR and rapamycin induced an increase in P-AMPK which was similar to that observed with AICAR alone, while P-mTOR inhibition was reversed by AICAR (Fig. 5a, b). However, no effect of AICAR was observed on P-S6 in the presence of both rapamycin and AICAR (Fig. 5c). Thirdly, we studied the effect of rapamycin and/or AICAR on ubiquitinated protein. Our results showed that rapamycin strongly increased ubiquitinated protein ($P < 0.01$) under both low and high AA concentrations in the presence of insulin (Fig. 5d). AICAR exerted no significant effect on protein ubiquitination at a low AA concentration, while it reversed the inhibition of protein ubiquitination induced by insulin at a high AA concentration, (Fig. 5d). Surprisingly, we observed that the effect of rapamycin on ubiquitinated proteins was reversed by AICAR (Fig. 5d) and returned to the level observed after inhibition by either high AA and/or insulin.

Discussion

This study assessed the adaptation to a high dietary protein intake of protein degradation in three tissues that play major roles in protein metabolism in rats, with a focus on the transition between the fasted and fed states. The results demonstrated tissue-specific responses of proteolysis to HP diets. In the liver, the HP diet was associated with an increase in the tissue protein pool, with a general trend for a more pronounced meal-induced inhibition of protein breakdown. By contrast, in peripheral tissues, no such changes were observed.

Previous studies described a stimulation of proteolysis over a long period of fasting of at least 24 h and an inhibition after feeding at the whole body level (Waterlow 2006), a regulation that is common to both muscle (Wing and Goldberg 1993; Wing and Banville 1994; Busquets et al. 2002) and liver (Mortimore et al. 1983; Mortimore et al. 1989; Bleiberg-Daniel et al. 1994). In the present study, when rats were fed a NP diet no significant meal-induced inhibition of protein degradation was observed after overnight fasting in the liver, kidney or muscle. Moreover, the significant meal-induced inhibition of proteolysis in the livers of rats fed the HP diet was in line with previous studies on whole-body protein turnover responses to increased protein intakes in humans (Pacy et al. 1994). Measures of whole body protein fluxes integrate different tissue fluxes, which may diverge one from another (Nair et al. 1995; Thivierge et al. 2005). The present results provide new insight by showing that tissue-specific responses to an HP diet can be observed using an ex vivo incubation method (Tischler et al. 1982) to measure these fluxes. The results indicated that the increased amplitude in diurnal N cycling observed with elevated protein intakes is more likely to concern specific protein pools such as hepatic proteins whereas muscle proteolysis is not significantly affected. The present study also indicates that the increased protein content in the liver previously observed

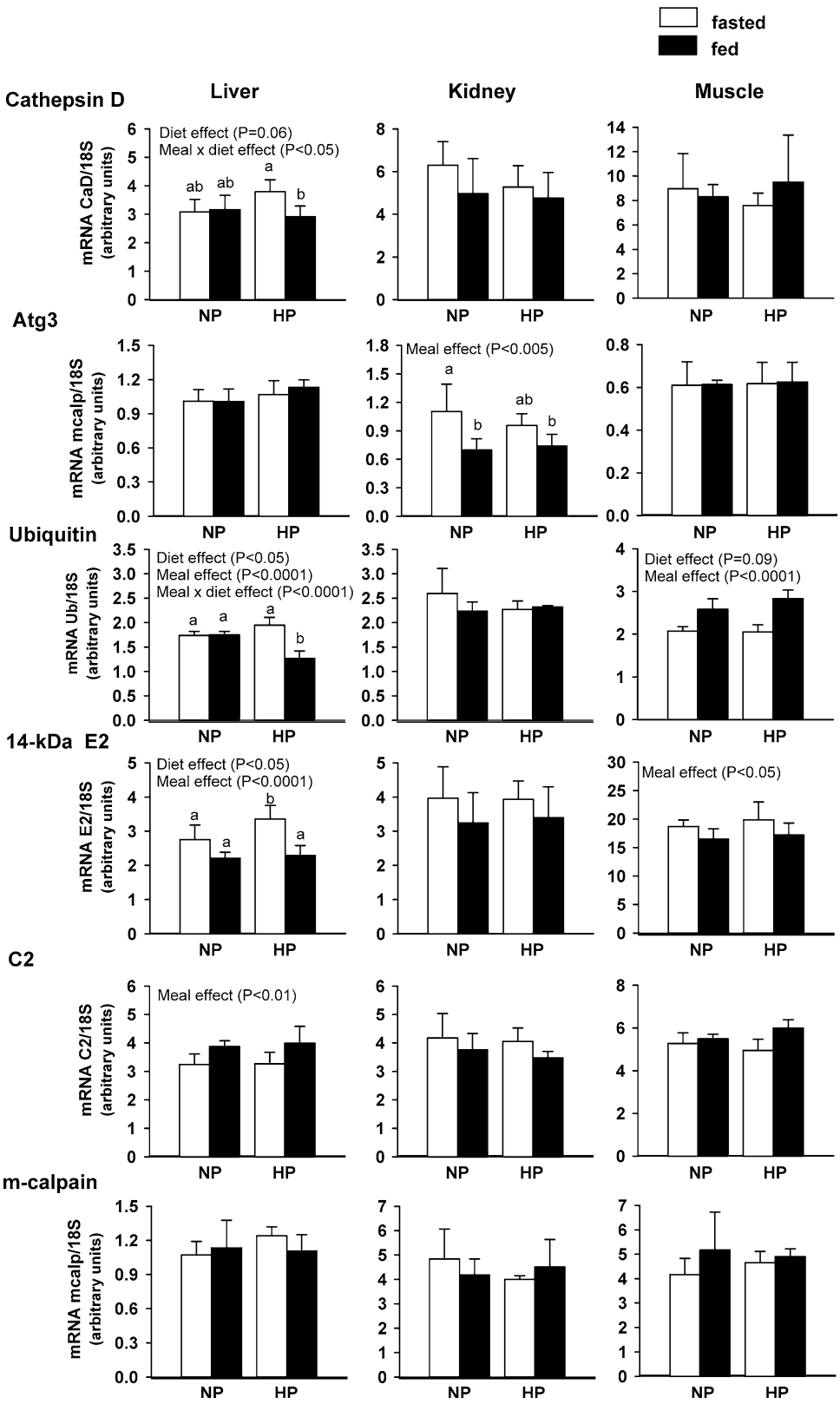

Fig. 2 Relative expression levels of genes encoding for the main proteolytic pathways in liver, kidneys, and muscle (soleus). The expression of mRNAs encoding for cathepsin D, Atg3, ubiquitin, 14-kDa E2 enzyme, C2 subunit of the proteasome and m-calpain were measured by real-time PCR in rats adapted to NP or HP diets for 14 days ($n = 24$) and sacrificed in the fasted or fed state. Values are mean ± SD. The effects of diet (NP or HP) or meal (fasted or fed) were assessed by two-way ANOVA. *Bars* with *different letters* within a graph are statistically different (post hoc Tukey tests for multiple comparisons, $P < 0.05$)

in HP-fed rats that could not be explained by an effect on liver protein synthesis was paradoxically reduced, (Chevalier et al. 2009) is in contrast directly related to a more pronounced meal-induced inhibition in protein breakdown. These adaptations of liver protein metabolism to the increase dietary protein in the diet suggest its

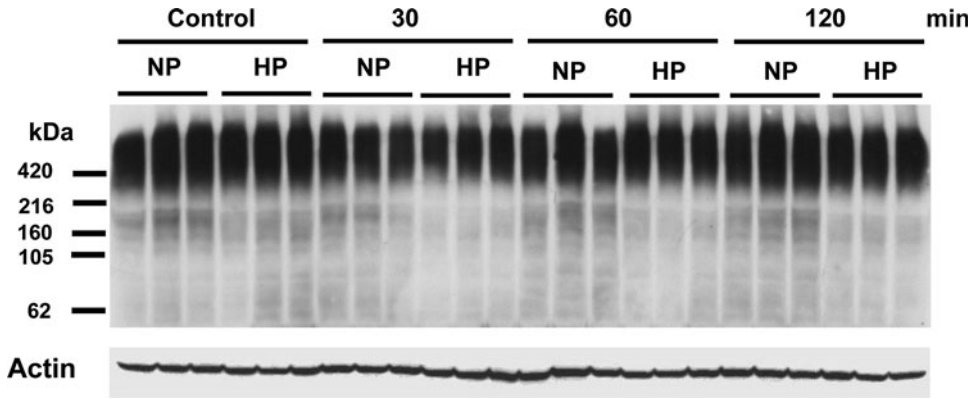

Fig. 3 Time course effect of NP and HP meals on protein ubiquitination. Protein extracted from the livers of rats adapted to NP or HP diets for 14 days ($n = 24$) and was processed for Western blot analysis as described in "Experimental design". The *upper* Western *blot* shows ubiquitinated protein in liver and the *bottom blot* shows the Western blot for actin. Only the bands between 216 and 62 kDa were quantified

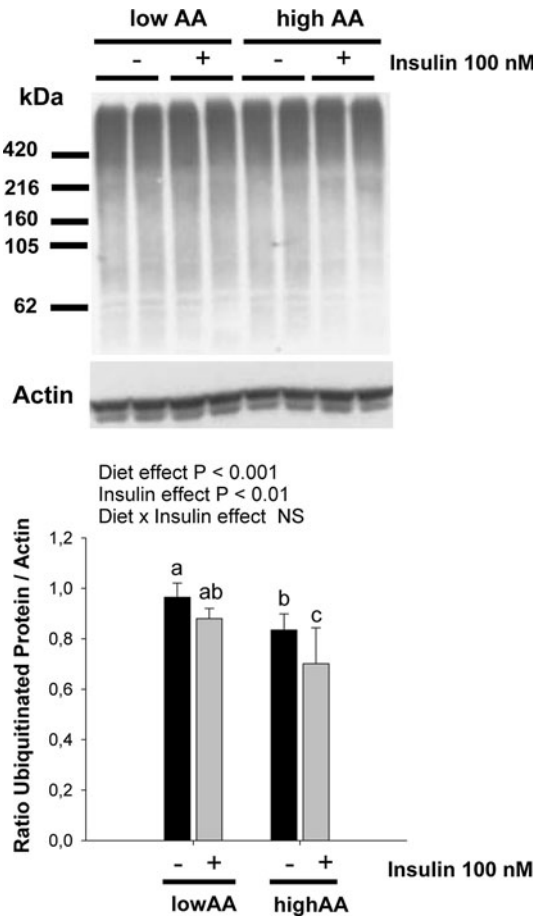

Fig. 4 Effect of amino acids (AA) and insulin on protein ubiquitination in a primary hepatocyte culture. Hepatocytes were incubated for 60 min in M199 salt medium containing 5 mM glucose plus low AA or high AA concentrations, with or without insulin. Protein extracts were processed for Western blot analyses. Results are representative of four separate experiments. The graphs represent the ratio of ubiquitinated protein and actin for eight samples from four separated cultures. Only the bands between 216 and 62 kDa were quantified. The results are expressed as mean ± SD, for $n = 8$. The effects of AA (low AA or high AA) or insulin (with or without insulin) were assessed by two-way ANOVA. *Bars* with *different letters* within a graph are statistically significantly different (post hoc Tukey tests for multiple comparisons, $P < 0.05$)

importance in protein homeostasis as in the case for carbohydrate and lipid homeostasis. Hence, the liver is more a regulatory organ, controlling the uptake and release of compounds to maintain energy homeostasis. Given that the liver is the only site for urea production, it provides a route for disposal of excess amino acids and their nitrogen content derived from dietary protein, in accordance with an old observation suggesting the existence of a labile proteins originally referred to by Lusk (1919). As for the other macronutrients, proteins can be stored in the liver providing the major source of oxidative fuel for the liver under high protein feeding both directly or through the conversion of amino acids in carbohydrate pathway (Stepien et al. 2010) without any adverse effects, particularly regarding hepatic histopathology (Lacroix et al. 2004).

The modulations affecting tissue protein degradation rates in response to an HP diet are consistent with the changes observed to the expression of major components in the proteolytic pathways, pointing to crucial roles for both autophagy and the ubiquitin–proteasome system in liver proteolysis. In the liver, ubiquitin and E2 enzyme expression was significantly inhibited by meal intake in HP-fed rats but not in NP-fed rats. As a consequence, liver proteins were less ubiquitinated and this process occurred as early as 30 min after the HP meal. The autophagic pathway may also be involved in these adaptations since cathepsin D expression was significantly lower in HP-fed rats than in HP-fasted rats. However, these variations did not differ from those seen in NP rats. These observations are in agreement with the role of the ubiquitin–proteasome dependent proteolytic pathway as a major cell catabolic process responsible for protein breakdown during short-term physiological and nutritional changes such as food deprivation (Ding et al. 1997). In the liver, the lysosomal pathway is generally considered to predominate (Bleiberg-Daniel et al. 1994; Blommaart et al. 1995; Del Roso et al. 2003; Kadowaki and Kanazawa 2003; Kanazawa et al. 2004), and only one study has reported a stimulation of hepatic ubiquitin expression after 1 or 2 days of starvation

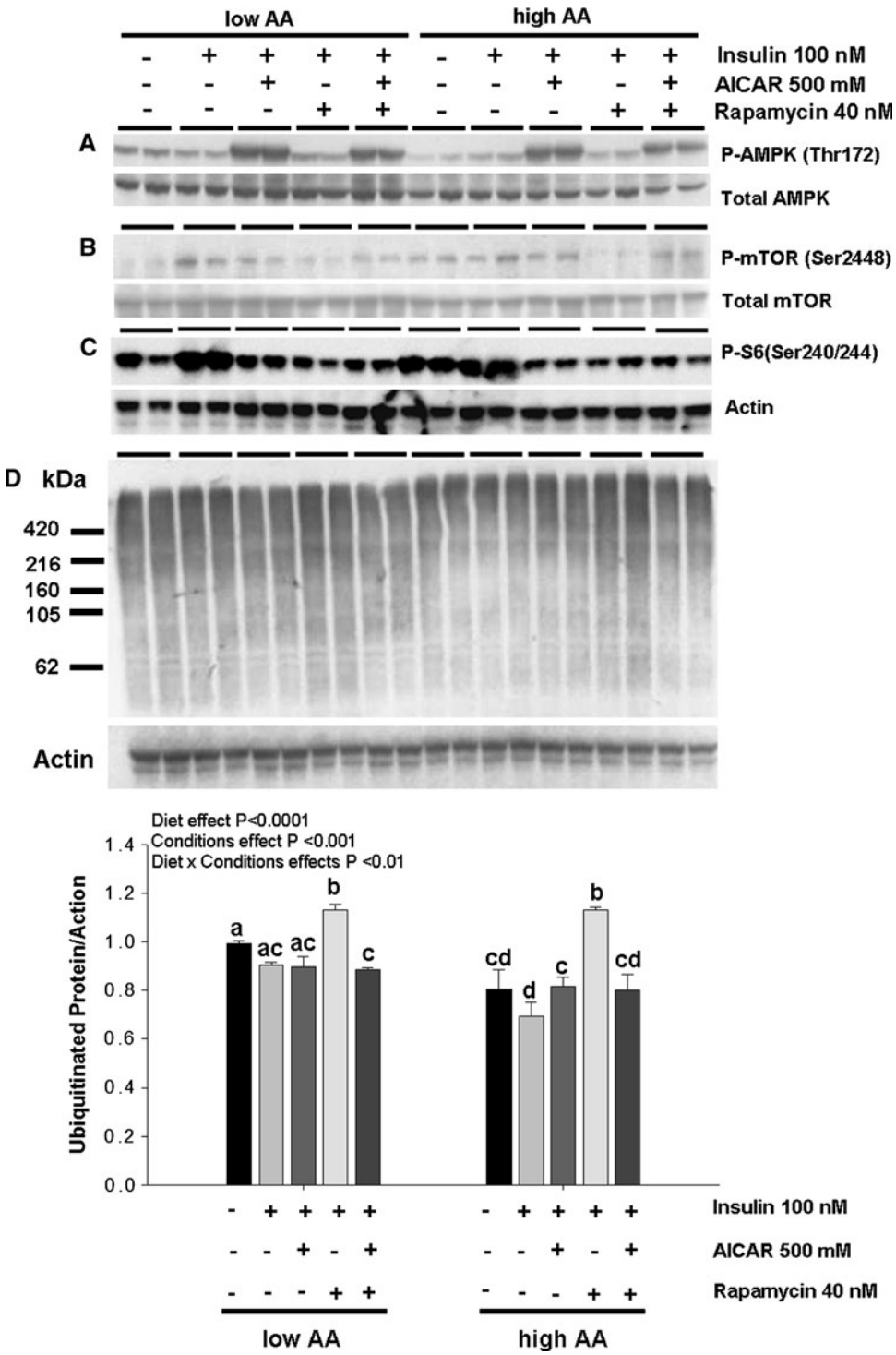

Fig. 5 The role of AMPK and mTOR in the control of protein ubiquitination in a primary hepatocyte culture. Hepatocytes were incubated for 60 min in M199 salt medium containing 5 mM glucose plus low or high amino acid (AA) concentrations, with or without insulin, AICAR and/or rapamycin. Protein extracts were processed for Western blot analysis. **a**, **b**, **c**, and **d** are representative of the Western blots for AMPKα phosphorylation (P-AMPKα) and total AMPKα (total AMPKα), mTOR phosphorylation (P-mTOR) and total mTOR (mTOR), S6 phosphorylation and actin and ubiquitinated protein and actin, respectively. For polyubiquitinated proteins, the bands between 216 and 62 kDa were quantified. Results are representative of two separate experiments. The graphs represent the ratio of ubiquitinated protein and actin for four samples from two separate cultures. The results are expressed as mean ± SD, for $n = 4$. The effects of AA (low AA or high AA) or conditions (with or without insulin, AICAR, and rapamycin) were assessed by two-way ANOVA. *Bars* with *different letters* within a graph are statistically significantly different (post hoc Tukey tests for multiple comparisons, $P < 0.05$)

(Wing and Banville 1994). In contrast to the liver, the ubiquitin–proteasome dependent proteolytic pathway is believed to play a major role in regulating protein degradation in muscle (Medina et al. 1991; Wing et al. 1995; Hamel et al. 2003), even if some recent data have suggested that autophagy and/or Ca^{2+} proteolysis are responsible for the postprandial inhibition of muscle protein breakdown (Capel et al. 2008). In muscle, moderate variations were seen to affect the expression of genes encoding key ubiquitin proteins, which was in line with the results regarding muscle protein breakdown. Numerous publications have described a stimulation of muscle protein breakdown in response to fasting, followed by an inhibition after re-feeding with a normal diet. The ubiquitin–proteasome pathway is the principal route of muscle protein breakdown that is activated in response to one or more days

of starvation (Medina et al. 1991; Wing and Banville 1994; Wing et al. 1995) Given that glucocorticoids are the main activators of the ubiquitin-dependant proteolytic system (Wing and Goldberg 1993; Marinovic et al. 2007), and that this hormone is released in response to long-term fasting, it could be expected that under short-term fasting, muscle proteolysis may not be activated as it was in the present study. In the kidney, a marked inhibition of Atg3 expression was observed in NP-fed rats that did not translate into consistent changes to the rate of proteolysis. Atg3 is one component in a large family of autophagy-related genes implicated in autophagosome formation (Levine and Klionsky 2004) a complex process that involves more than 16 components. The inhibition of Atg3 observed in the study, together with the absence of changes to the expression of other components in the autophagy pathway, suggests that Atg3 alone may not be a good predictor of proteolytic fluxes.

Insulin is known to be involved in the fed-state inhibition of protein breakdown (Gelfand and Barrett 1987), and liver proteolysis is known to be inhibited by insulin and stimulated by glucagon (Schworer and Mortimore 1979; Mortimore et al. 1989). Plasma insulin levels are generally lower or similar in the fed state in rats or humans subjected to a HP/low carbohydrate diet, whereas glucagon concentrations is increased (Lacroix et al. 2004; Harber et al. 2005; Baum et al. 2006; Blouet et al. 2006; Azzout-Marniche et al. 2007). Thus, the stronger inhibition of hepatic proteolysis induced by an HP diet could not be ascribed directly to insulin. Plasma amino acid and branched-chain amino acid concentrations are increased by HP diets in the fed state (Forslund et al. 1998; Blouet et al. 2006; Azzout-Marniche et al. 2007). Moreover, the results showed that the increased amino acid concentration in the media was sufficient to induce a decrease in ubiquitinated protein in a primary culture of hepatocytes, and that this inhibition was stronger in the presence of insulin. This result agrees with previous findings which demonstrated an inhibitory effect of different amino acid mixtures on protein degradation in the perfused rat liver (Poso et al. 1982; Mortimore et al. 1989; Kadowaki et al. 1992; Miotto et al. 1992) and isolated hepatocytes (Seglen et al. 1980).

It has been proposed that control of the anabolic and catabolic pathways by amino acids may be coordinated by the same signaling system and might involve the mTOR pathway (Blommaart et al. 1995). Moreover, it has previously been demonstrated that both amino acids and insulin are required to stimulate translation and are involved at least in the mTOR, AMPK, and GCN2 transduction pathways (Chotechuang et al. 2009). We observed that rapamycin, the mTOR inhibitor, stimulated protein ubiquitination under both low and high amino acid levels in the presence of insulin. It has been reported that in hepatocytes, rapamycin prevents the inhibition of autophagic proteolysis by insulin and amino acids during a lengthy exposure (60 and 90 min) (Blommaart et al. 1995; Kanazawa et al. 2004). This suggests that in the liver, the mTOR transduction pathway, as well as regulating autophagy, is also involved in controlling the ubiquitin–proteasome system in response to a high protein intake. Surprisingly, we observed that AICAR reversed the inhibition of protein ubiquitination induced by insulin under high amino acid levels, while it had no effect at low amino acid levels. Moreover, the effect of rapamycin on protein ubiquitination was abolished in the presence of AICAR. These findings suggest that the inhibition of AMPK and the activation of mTOR transduction pathways were required for the down-regulation of protein ubiquitination in response to high amino acid and insulin concentrations. It is clear that the modification made to mTOR activity by rapamycin had no effect on AMPK phosphorylation state. By contrast, it not clear how mTOR phosphorylation was affected by AMPK activation, because we observed that the latter reversed mTOR inhibition by rapamycin. AMPK can directly phosphorylate mTOR on Thr^{2446}, leading to its inactivation (Cheng et al. 2004), or indirectly via phosphorylation of the tuberous sclerosis complex (TSC) two-gene product (TSC2) and raptor (Gwinn et al. 2008). Furthermore, mTOR full activation requires mTORC1 complex formation (mTOR, raptor, PRAS40, mLST8), and amino acids stimulate the relocation of mTOC1 to its correct site (Sancak et al. 2008; Shaw 2008). However, we cannot exclude the possibility that the activation of AMPK targets a protein downstream in mTOR signaling. One of the best candidates is the Forkhead proteins. In the liver, insulin, through Akt signaling, phosphorylates Foxo proteins leading to their exclusion from the nuclei. The further consequences are first the polyubiquitination and degradation of Foxo proteins, and second, the disturbance of the binding to Foxo of its coactivator, PGC-1 alpha (Mounier and Posner 2006). In muscles, two Foxo-induced genes are particularly important in enhancing proteolysis, two muscle-specific ubiquitin ligases (UL). In C2C12 myotubes, mTOR inhibition by rapamycin suppressed the IGF-1 induced inhibition of muscle-specific UL whereas AMPK activation by AICAR stimulates UL expression, which may be due to a possible antagonistic effect of Foxo phosphorylation by AICAR (Tong et al. 2009). Similar mechanisms may be involved in the regulation of Foxo proteins and ubiquitin ligase in the liver. It remains necessary to clarify whether AMPK is coordinated with mTOR in the regulation of protein ubiquitination, as both are known to act with GCN2 in order to control translation (Chotechuang et al. 2009).

The present results thus provide evidence of a major role for liver proteolysis in the control of protein accretion in response to an increased dietary protein intake in rats. The ubiquitin–proteasome system and autophagy are the

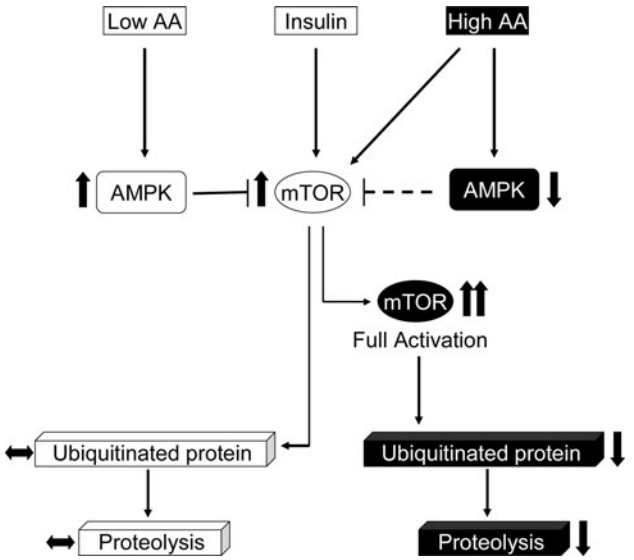

Fig. 6 Proposed scheme for the coordinated action of insulin and amino acids on hepatic ubiquitin-dependent proteolysis. When the AA concentration is low, insulin stimulates the phosphorylation of mTOR but without any significant effect on the proteolysis rate and the ubiquitin protein breakdown pathway. When the amino acid concentration increases, ubiquitinated protein is reduced when compared with a low amino acid concentration and this effect is stronger in the presence of insulin. The mTOR transduction pathway plays a key role in control of the ubiquitin–proteasome system. The inhibition of mTOR by rapamycin reverses the inhibition of protein ubiquitination under low and high amino acid levels in the presence of insulin, which suggests that stimulation of the mTOR pathway is required for inhibition of the ubiquination process. The effects of insulin and amino acids on mTOR full activation require: (1) inactivation by insulin of the TSC complex (tuberous sclerosis complex of TSC1/TSC2), the negative regulator of mTOR, (2) mTORC1 complex formation (mTOR, raptor, PRAS40, mLST8) and (3) the amino acid stimulation of mTORC1 relocation. Moreover, the AMPK transduction pathway is also involved in this process. AMPK activation by AICAR reverses inhibition of the ubiquitination process induced by an increase in the AA concentration whereas it has no significant effect at a low AA concentration in the presence of insulin. Interestingly, AICAR reverses the effect of rapamycin with both low and high AA concentrations, suggesting that a decrease of P-AMPK is required for activation of the ubiquitination proteolysis pathway by mTOR inhibition. Moreover, mTOR phosphorylation is affected by AICAR, which suggests that AMPK controls mTOR phosphorylation, either directly by phosphorylation or indirectly through subcellular localization of the mTOR complex

principal pathways involved in this process. Furthermore, in hepatocytes, an increase in the amino acid concentration was sufficient to down-regulate the ubiquitin–proteasome pathway, but this inhibition was stronger in the presence of insulin. The mTOR signaling pathway may be involved in the down-regulation of protein ubiquitination in response to a HP diet. A proposed scheme for the coordinated control by insulin and amino acids of liver ubiquitin protein breakdown as a function of the protein level in the diet, is shown in Fig. 6. In many tissues, proteolysis assumes an essential cellular homeostatic or housekeeping function, removing damaged or unwanted organelles and proteins. It has been proposed that the homeostatic function of autophagy represents an anti-aging mechanism and may be involved in the conserved effect of protein caloric restriction (Del Roso et al. 2003). A clearer understanding of the nutritional control of protein breakdown regulation, and particularly the effect of protein intake, would be of importance in the context of preserving muscle mass during aging.

Conflict of interest None.

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