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Journal of Nutritional Biochemistry 23 (2012) 1467–1473

Journal of Nutritional Biochemistry

Regulators of protein metabolism are affected by cyclical nutritional treatments with diets varying in protein and energy content☆

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Received 3 May 2011; received in revised form 12 September 2011; accepted 22 September 2011

Abstract

There is evidence that the E3 ubiquitin ligases muscle ring finger-1 (MuRF1) and atrogin-1, which mediate the ubiquitination of certain proteins and thereby their proteolysis, are regulated by cyclical nutritional treatments varying in lysine content. In order to explore further the regulatory mechanisms involved in metabolic adaptation to dietary changes, we investigated the effects of daily variations in energy [2800 (E−) followed by 3200 kcal/kg (E+)], protein [230 (P+) followed by 150g/kg (P−)] or both [E−P+ followed by E+P−] on muscle protein metabolism in 2-week-old male broiler chickens. Growth performance was similar for all treatments. Expression of atrogin-1 and MuRF1 was changed by alternation of diets varying in protein (higher expression with P− vs. P+) and energy content (higher expression with E− vs. E+). The expression of atrogin-1 was regulated with mixed diets (increase in E+P− vs. E−P+) but not that of MuRF1. Such regulation may involve the mammalian target of rapamycin (mTOR), which was more phosphorylated with P+ than with P−. Eukaryotic initiation factor 4E binding protein, p70S6 kinase and ribosomal protein S6, which are mTOR targets known to control protein synthesis, were highly activated by increased protein content (P+ vs. P−). The mechanisms coordinating the protein synthesis/proteolysis balance remain to be characterized.

Keywords: Sequential feeding; Protein; Energy; Metabolism; Chicken muscle

1. Introduction

The nutritional regulation of metabolism involves feeding level, the nature of nutrients and the rate of nutrient delivery [1–4]. There is evidence that the timing of protein feeding regulates protein metabolism. For instance, a pulse protein feeding pattern (i.e., consuming the greater part of daily proteins in a single meal compared to distribution of daily proteins in a number of meals) results in a higher nitrogen balance in the elderly and in older animals because of lower leucine oxidation and whole-body protein degradation during the postabsorptive state and higher stimulation of protein synthesis in the whole body, liver and muscle during the fed state [5,6]. Similarly, the kinetics of digestion of proteins and the resulting amino acid absorption from the gut have a major effect on protein anabolism. Indeed, studies in humans indicate that slowly absorbed proteins (e.g., casein) may promote overall protein deposition by reducing protein degradation [7], a result obtained whether slowly absorbed proteins are offered without or simultaneously with nonprotein energy. However, when energy is also provided, quickly absorbed proteins can also be efficient [7–9], demonstrating the importance of taking into account both protein and energy supply when exploring time-dependent regulation of protein metabolism.

Recent findings obtained in chickens suggest temporal regulation of muscle proteolysis according to dietary treatment and a high metabolic capacity to compensate for cyclical changes in amino acid supply [10]. More precisely, genes related to proteolysis, in particular, the E3 ubiquitin ligases muscle atrophy F box (MAFbx or atrogin-1) and muscle ring finger-1 (MuRF1) are overexpressed in the skeletal muscle of chickens transiently receiving lower lysine intake in sequential feeding programs. These two important genes (also called atrogenes) control the polyubiquitination and thus the degradation of certain proteins, including myofibrillar proteins [11–13]. They are regulated by hormones such as insulin and amino acids via mechanisms involving the forkhead box-O transcription factor (FoxO) and the mammalian target of rapamycin (mTOR) pathways [14–17]. mTOR is also involved in the control of protein synthesis

Abbreviations: atrogin-1 or MAFbx, muscle atrophy F box; C2, proteasome C2 subunit; Cs, ribosomal capacity; 4E-BP1, eukaryotic initiation factor 4E binding protein; FoxO, forkhead box-O transcription factor; mTOR, mammalian target of rapamycin; MuRF1, muscle ring finger-1; S6K1, 70 kDA ribosomal protein S6 kinase; S6, ribosomal protein S6.

☆ Supported by research grants from ACTA/MAP/MESR and INRA.

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doi:10.1016/j.jnutbio.2011.09.009

through its downstream targets p70 S6 kinase (S6K1) and the translational repressor eukaryotic initiation factor 4E binding protein (4EBP1) (see Kimball and Jefferson [18] and Tesseraud et al. [19] for reviews). 4EBP1 corresponds to the binding protein of initiation factor eIF4E and acts as a competitive inhibitor of eIF4F complex formation, subsequently limiting the formation of the 48S preinitiation complex. S6K1 is a serine/threonine kinase that phosphorylates several proteins, including the ribosomal protein S6, also affecting the initiation stage of mRNA translation. To our knowledge, little has been established regarding the mechanisms controlling the protein synthesis/proteolysis balance in response to cyclical changes in dietary supply. The aim of the present study was therefore to explore the potential changes in protein metabolism and signaling in chicken submitted to sequential feeding with diets varying in protein and/or energy content. The experiment was designed to distinguish the respective effects of protein and energy content.

2. Methods and materials

2.1. Broiler chickens and distribution of feeds

A total of 720 1-day-old male broiler chicks (Ross PM3) were wing-banded and randomly distributed into 36 pens (1.6×1.75 m, 20 chicks per pen) in a conventional environmentally controlled poultry shed at the Poultry Research Centre in Nouzilly, France. The floor was covered with wood shavings. Lighting was reduced from 24 light (L):0 dark (D) to 14L:10D daily after the age of 2 days and was then increased to 16L:8D daily at the age of 7 days. Environmental temperature was progressively reduced from 32°C to 23°C. Feed was provided in linear feeders. All the chickens received the same starter diet (2900 kcal/kg metabolizable energy (ME), 21% crude protein (CP)). From the age of 10 days, chickens were given either control (C) or sequential programs *ad libitum*.

Seven diets (Table 1) were used for the sequential programs, as previously proposed by Bouvarel et al. [20], with the applied goal of developing new nutritional strategies or modifying constraints for diet formulation. The control diet was complete feed C (3000 kcal/kg ME, 19.5% CP). Two diets were isoenergetic with differences in protein and essential amino acid content: the high-protein diet (P+, 23.4% CP) and the low-protein diet (P−, 15.6% CP). Two diets were isoproteic with differences in ME content: the low-energy diet (E−, 2800 kcal/kg ME) and the high-energy diet (E+, 3200 kcal/kg ME). Two diets had differences in both protein and energy content: the high-protein and low-energy diet (E−P+) and the low-protein and high-energy diet (E+P−). Three sequential feeding treatments were used: (1) alternation of diets varying in crude protein (S_P, P+ followed by P−), (2) alternation of diets varying in energy (S_E, E− followed by E+) and (3) alternation of diets varying in protein and energy content (S_{EP}, E−P+ followed by E+P−). Each "new" diet was introduced after the dark phase, during which chickens were not eating.

Table 1
Composition (%) and characteristics of experimental feeds

	C	S_P		S_E		S_{EP}	
Ingredients		P+	P−	E−	E+	E−P+	E+P−
Corn	20.12	31.00	30.54	20.58	45.23	12.35	50.44
Wheat	50.00	24.78	50.00	50.00	20.96	50.00	27.61
Soybean meal	18.39	31.48	12.05	24.78	17.87	27.06	9.00
Corn gluten	4.73	5.00	1.48	0.18	7.00	6.00	5.00
Rapeseed oil	2.91	4.00	2.14	0.80	5.00	0.77	4.00
Dicalcium phosphate	1.34	1.46	1.37	1.32	1.54	1.29	1.54
Calcium carbonate	1.24	1.07	1.27	1.21	1.12	1.21	1.19
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Lysine HCl	0.37	0.27	0.31	0.21	0.39	0.36	0.39
DL-Methionine	0.14	0.21	0.09	0.17	0.11	0.21	0.05
Tryptophan	0.000	0.000	0.000	0.000	0.023	0.000	0.028
Premix *	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Calculated content							
ME (kcal/kg)	3000	3000	3000	2800	3200	2800	3200
CP (%, analyzed)	19.50	23.40	15.60	19.60	19.40	23.40	15.70
Fat (%, analyzed)	5.1	6.3	4.5	2.9	7.6	3.0	6.8
ME:CP (kcal/g CP)	15.4	12.8	19.2	14.3	16.5	12.0	20.4
Lysine (%)	1.07	1.29	0.84	1.07	1.07	1.29	0.84
TSAA (%)	0.81	0.99	0.64	0.81	0.81	0.99	0.64
Threonine (%)	0.67	0.84	0.53	0.68	0.68	0.81	0.53
Tryptophan (%)	0.20	0.25	0.16	0.22	0.20	0.25	0.16
Calcium (%)	0.95	0.95	0.95	0.95	0.95	0.95	0.95
Available phosphorus (%)	0.37	0.37	0.37	0.37	0.37	0.37	0.37

TSAA indicates total sulfur amino acids.
* Composition as previously described by Bouvarel et al. [20].

Sequential feeding was carried out over 48-h cycles. Experiments on sequential feeding using 48-h cycles previously showed that this technique had no negative effect on performance if the consumption of the sequential feeds provided an energy and amino acid intake similar to the control diet [20,21]. In the present study, on days 14 and 15 (cycle 3), six chickens per diet from different pens were used for measurements, corresponding to chickens receiving the complete diet (C), the P+ diet (S_P sequential program), the E− diet (S_E sequential program) and the E−P+ diet (S_{EP} sequential program) on day 14 and to chickens receiving the complete diet (C), the P− diet (S_P sequential program), the E+ diet (S_E sequential program) and the E+P− diet (S_{EP} sequential program) on day 15. These chickens were weighed at 9 days of age and at the end of the experiment (on days 14–15). Throughout this period, chickens from all feeding programs (i.e., all sequential programs and control diet) showed similar growth rates (40.0 ± 1.4 g/d). Feed intake per pen calculated for the remaining birds was not significantly different between feeding programs (56.8 ± 1.3 g/d per chicken during the three cycles corresponding to 10–15 days of age).

Approximately 5 h after the changes of diet that took place at 9:00 a.m. each day, chickens used for measurements were sacrificed by decapitation after blood sampling. The pectoralis major muscles were removed and quickly frozen. Frozen tissues were ground in liquid nitrogen and stored at −80°C until analysis. All experiments were carried out with due regard to legislation governing the ethical treatment of animals, and investigators were certified by the French government to carry out animal experiments.

2.2. Plasma parameters and muscle characteristics

Plasma αNH2 NP N concentrations (α-amino-nonprotein nitrogen, an estimate of total free amino acids) were measured after extraction with 10% (v/v) sulfosalicylic acid using 2% ninhydrin reagent (Sigma Chemicals) and L-serine as standard [22]. Plasma insulin levels were determined by radioimmunoassay with a guinea pig anti-porcine insulin antibody (Ab 27-6, generously provided by Dr. G. Rosselin, Saint-Antoine Hospital, Paris, France) using chicken insulin as the standard [23]. All samples were run in the same assay in order to avoid interassay variations.

For measurement of muscle protein, RNA and ribosomal capacity, frozen powdered muscle samples were homogenized in 2% HClO4 according to the method of Schmidt-Thannhauser as modified by Munro and Fleck [24]. Protein content was measured according to Smith et al. [25] by colorimetric reaction with bicinchoninic acid. Total RNA was measured on the basis of the ultraviolet absorbance at 260 nm, with a correction for peptide material based on the ultraviolet absorbance at 232 nm. Ribosomal capacity, i.e., the capacity for protein synthesis (an index related to the number of ribosomes and thus protein synthesis machinery), was calculated as the ratio of RNA to protein [26].

2.3. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNA Now (Biogentec, Seabrook, TX, USA) from 100-mg muscle samples according to the manufacturer's recommendations. After RNase-Free DNase treatment, RNA was reverse-transcribed using Super Script II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in the presence of Random Primers (Promega, Charbonnieres-les-Bains, France). Quantitative PCRs were performed in duplicate using the Abi Prism 7000 apparatus (Applied Biosystems, Foster City, Ca, USA) as previously described [27]. The primers chosen to amplify parts of MuRF1, atrogin-1, m-calpain, cathepsin B, 20S proteasome C2 subunit and ubiquitin had previously been used in our laboratory [10]. Gene expression levels were estimated on the basis of PCR efficiency and threshold cycle deviation of an unknown sample versus a control, as previously described [27]. 18S ribosomal RNA was chosen as the reference gene.

2.4. Peptidase activity of the proteasome

Frozen powdered muscle samples were homogenized in ice-cold buffer (pH 7.5) containing 50 mmol/L Tris, 250 mmol/L sucrose, 10 mmol/L ATP, 5 mmol/L MgCl2, 1 mmol/L DTT and protease inhibitors. As previously described [28], the proteasomes were isolated by three sequential centrifugations, and the final pellet was resuspended in buffer containing 50 mmol/L Tris (pH 7.5), 5 mmol/L MgCl2 and 20% glycerol. Peptidase activity of the proteasome was determined from 15 μg of proteins of the proteasome preparation by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY) and Boc-Leu-Arg-Arg-7-amido-4-methylcoumarin (LRR) (Sigma, Saint Louis, MO, USA). These substrates are preferentially hydrolyzed by the chymotrypsin-like and the trypsin-like activity of the proteasome, respectively. Peptidase activity was determined by measuring the accumulation of the fluorogenic reagent methylcoumarylamide (AMC) using an LS50B luminescence spectrometer (Perkin Elmer, Waltham, MA, USA) (excitation and emission wavelengths of 360 nm and 430 nm, respectively).

Table 2
Feed intake in chickens receiving either control (C) or sequential programs at 14 and 15 days of age *

Sequential treatment †	C		S_P		S_E		S_{EP}		S.E.M.	P <
Diet	C	C	P+	P−	E−	E+	E−P+	E+P−		
Day of experiment ‡	d14	d15	d14	d15	d14	d15	d14	d15		
Average feed intake, g/d	65.4		64.0		63.4		62.8		1.9	NS
Daily feed intake, g/d §	64.1[ab]	66.7[a]	59.9[bc]	68.2[a]	57.3[c]	69.4[a]	55.3[c]	70.2[a]	1.9	<.05

* Feed intake was calculated per pen and expressed per chicken. Results are expressed as means ± S.E.M. (n=9). They were analyzed using one-way ANOVA. Means not sharing the same letter are significantly different (P <.05).
† S_P, sequential feeding with alternation of diets varying in crude protein (P+ followed by P−); S_E, sequential feeding with alternation of diets varying in energy (E− followed by E+); S_{EP}, sequential feeding with alternation of diets varying in protein and energy content (E−P+ followed by E+P−).
‡ Day of experiment: d14 (first day of the cycle 3) and d15 (second day of the cycle 3).
§ Daily feed intake: consumption of each diet over the 24 h of distribution.

2.5. Western blotting

To analyze the signaling pathways potentially involved in regulation of protein metabolism, muscle lysates were prepared as previously described [29]. Muscle lysates (60 μg of protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting using the appropriate antibodies: p-FoxO1 [T24] and p-FoxO3a [T32], p-mTOR [S2448], p-S6K1 [T389], p-S6K1 [T421/S424], p-S6 [S235/S236] and p-4EBP1 [S65] (Cell Signaling Technology, Beverly, MA, USA); S6K1 and mTOR/FRAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and vinculin (Sigma Chemical Company). After washing, membranes were incubated with an Alexa Fluor secondary antibody (Molecular Probes, Interchim, Montluçon, France). Bands were visualized by Infrared Fluorescence by the Odyssey Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE, USA) and quantified by Odyssey infrared imaging system software (Application software, version 1.2).

2.6. Statistical analysis

Values are presented as means ± S.E.M. Data were processed using the Statview Software program, version 5 (SAS Institute, Cary, NC, USA). The homogeneity of variance between groups was tested by Bartlett's test. In the case of heterogeneity of variance (for peptidase activity of the proteasome), the nonparametric Kruskal–Wallis test was used to test differences between groups, and means were compared using the Mann–Whitney test. For all other parameters, data were subjected to analysis of variance (ANOVA) to detect significant intergroup differences. The means were compared by Fisher's least significant difference test in the case of a significant effect. P<.05 was considered statistically significant.

3. Results

3.1. Animal characteristics

Within the sequential feeding cycle 3 (i.e., on days 14–15), the average feed intake calculated per pen and expressed per chicken did not show any significant differences between feeding programs (63.9 ± 1.8 g/d) (Table 2). In the sequential feeding treatments S_P, S_E and S_{EP}, daily feed intake was higher on day 15 than on day 14, whereas it was not significantly different between diets at each time point (i.e., at 14 and 15 days of age).

Five hours after switching diets, daily dietary changes did not drastically modify plasma insulin concentration (2.70 ± 0.44 ng/ml) (Table 3). The plasma levels of total free amino acids were significantly higher with the P+ diet compared to the P− diet (2.1-fold higher, P<.05). We did not observe any significant difference in the levels of free amino acids between E+ and E− diets and between E−P+ and E+P− diets. There was no significant difference between groups with regard to pectoralis major muscle RNA and protein content (Table 3). The capacity for muscle protein synthesis (i.e., RNA/protein ratio) was thus not changed whatever the diet.

3.2. Daily changes in dietary protein and/or energy content modulated MuRF1 and atrogin-1 expression in chicken muscle

Five hours after switching diets, we explored the potential regulation of atrogin-1 and MuRF1 by daily variations in energy or protein, or both (Fig. 1A). The levels of mRNA expression of atrogin-1 and MuRF1 were dramatically affected by the alternation of diets varying in protein, with overexpression with P− compared to P+ (4.5-fold for atrogin-1, P<.001; 1.6-fold for MuRF1, P<.01). Atrogin-1 and MuRF1 were underexpressed with E+ compared to E− (2.7-fold for atrogin-1 and 1.8-fold for MuRF1, P<.01). With a mixed diet sequence, the expression of atrogin-1 was up-regulated in E+P− compared to E−P+ (2.1-fold, P<.05), but not that of MuRF1. On the other hand, the expression of certain other genes related to proteolysis (i.e., cathepsin B, m-calpain, ubiquitin and 20S proteasome C2) was not modified by any treatment (data not shown). To explore further the potential changes in the ubiquitin proteasome system, we studied the activity of the major proteasome peptidases (i.e., trypsin-like and chymotrypsin-like) in the pectoralis major muscle. Peptidase activity of the proteasome was not drastically changed by the alternation of diet, with the exception of the mixed diet sequence, where levels of trypsin-like and chymotrypsin-like

Table 3
Effects of daily changes in dietary protein and/or energy content on plasma parameters and muscle characteristics in chickens at 14–15 days of age *

Sequential treatment †	C	S_P		S_E		S_{EP}		S.E.M.	P <
Diet	C	P+	P−	E−	E+	E−P+	E+P−		
Plasma parameters									
Insulin, ng/ml	3.58	3.28	2.59	2.63	3.22	2.80	2.69	0.38	NS
αNH2 NP N, mg/ml ‡	0.89 [a]	0.98 [a]	0.57 [b]	0.76 [ab]	0.88 [a]	0.91 [a]	0.73 [ab]	0.07	<.05
Muscle characteristics									
Protein, mg/g	115	114	112	106	117	116	118	5	NS
RNA, μg/g	1.99	2.00	1.88	1.90	2.01	1.99	2.05	0.07	NS
Cs, mg RNA/g protein §	17.4	17.6	16.9	18.0	17.2	17.3	17.5	0.6	NS

* Measurements were performed 5 h after the switch of diet. Results are expressed as means ± S.E.M. (n=6 except for C where n=12). They were analyzed using one-way ANOVA. Means not sharing the same letter are significantly different (P <.05).
† S_P, sequential feeding with alternation of diets varying in crude protein (P+ followed by P−); S_E, sequential feeding with alternation of diets varying in energy (E− followed by E+); S_{EP}, sequential feeding with alternation of diets varying in protein and energy content (E−P+ followed by E+P−).
‡ αNH2 NP N, αNH2 nonprotein nitrogen, an estimate of free amino acids, is expressed as mg L-serine equivalent/ml.
§ Cs, capacity for protein synthesis.

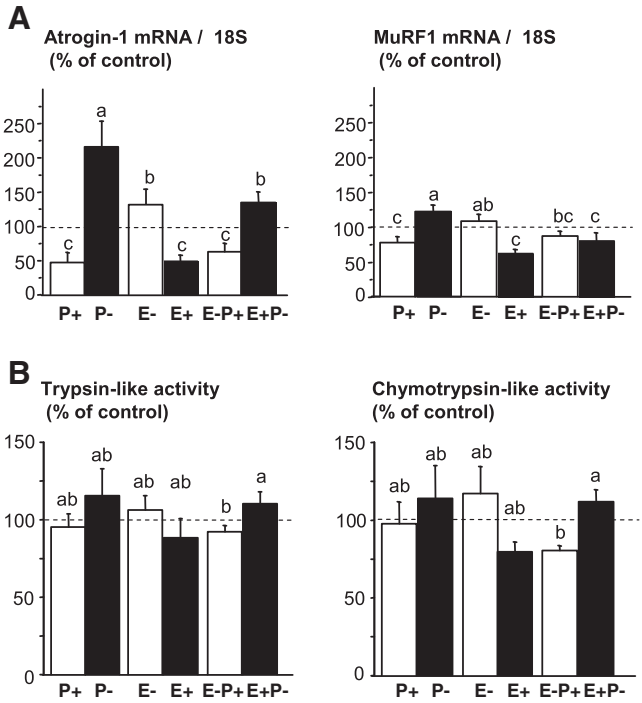

Fig. 1. Effects of daily changes in dietary protein and/or energy content on the expression of atrogin-1 and MuRF1 (A) and on trypsin-like and chymotrypsin-like peptidase activity of the proteasome (B) in breast muscle of 2-week-old chickens. Measurements were performed 5 h after the switch of diet. Analyses were carried out by real-time RT-PCR. mRNA levels were normalized using 18S rRNA; 18S was not significantly affected by dietary treatments. Results are expressed as percentages of the control on the day of the experiment and presented as means ± S.E.M. (n=6). Means not sharing the same letter are significantly different (P<.05).

activity were higher in E+P− than in E−P+ (1.2-fold and 1.4-fold, respectively; P<.05) (Fig. 1B).

3.3. TOR responded to daily changes in dietary protein and/or energy content

We first focused on the changes in FoxO phosphorylation potentially induced by daily variations in dietary protein and energy content. The levels of phosphorylated FoxO1 and FoxO3a measured 5 h after the switch of diet were not significantly changed by sequential feeding (Fig. 2A). Since mTOR may also be involved in the regulation of E3 ubiquitin ligases, we next measured the phosphorylation of mTOR (Fig. 2B). The level of mTOR phosphorylation was significantly greater in the pectoralis major muscle of chickens fed the P+ diet compared to the P− diet (1.7-fold, P<.05). The difference in mTOR phosphorylation between the E−P+ and E+P− diets was not significant, and fairly similar values were observed in the breast muscle of chickens fed the E− and E+ diet.

3.4. mTOR targets related to mRNA translation were also regulated

To assess whether mTOR targets related to mRNA translation into proteins were regulated, we next examined the downstream effectors S6K1, S6 and 4EBP1. We explored the phosphorylation of S6K1 on the serine/threonine residues considered to be most critical for kinase function, i.e., T389 and T421/S424 [30,31]. The level of S6K1 phosphorylation was significantly greater in the breast muscle of chickens fed the P+ diet compared to the P− diet (4.5- to 5.1-fold according to serine/threonine residue, P<.05) (Fig. 3A). Similarly, S6 and 4EBP1 were more highly phosphorylated in the muscle of chickens fed the P+ diet compared to the P− diet (4.2- and 1.85-fold for S6 and 4EBP1, respectively; P<.05) (Fig. 3B–C). The levels of phosphorylated S6K1 and 4EBP1 measured 5 h after the switch of diets differing in energy content were not significantly different. Nevertheless, the E+ diet led to increased S6 phosphorylation compared to the E− diet (3.5-fold, P<.05). Alternation of mixed diets resulted in relatively more consistent findings: although there was no significant difference between E−P+ and E+P− diets for S6K1 phosphorylation on T389, higher levels of p-S6K1 on T421/S424 (2.6-fold, P<.05), p-S6 (1.7-fold, P=.06) and p-4EBP1 (1.7-fold, P<.05) were found in the E−P+ diet compared to the E+P− diet.

4. Discussion

Sequential feeding programs with daily variations in dietary protein and/or energy content were carried out to explore the short-term regulation of protein metabolism in chicken skeletal muscle. We used 48-h cycles that did not impair overall growth performance as previously demonstrated [20,21] or capacity for protein synthesis (i.e., protein synthesis machinery) in chicken pectoralis major muscles. Chickens from all feeding programs exhibited similar average feed intake. Overconsumption was found on the second day of the cycle, which can be explained by the general increase in daily feed intake related to chicken growth and potential changes in feed preferences. Clearly, similar sequential feeding experiments [32] showed a notable effect measured in the short term with diets varying in energy. Birds decreased their consumption of low-energy diets during the first 15 min of distribution and conversely increased consumption of high-energy diets. These experiments did not reveal any effect on feed intake with diets varying in protein content.

In these experimental conditions, alternation of diets varying in protein and energy content applied for two cycles resulted in marked regulation of atrogin-1 and MuRF1. Such regulation of the two E3 ubiquitin ligases in response to dietary provision is in good agreement with the results obtained using another 48-h cycle sequential feeding program with diets varying in lysine content for seven cycles [10]. This implies overexpression of atrogin-1 and MuRF1 in the breast muscle of chickens transiently receiving lower provision of protein, amino acid (i.e., lysine) and energy, whatever the study. It should be noted that this effect was found without lower quantities of P− consumed (vs. P+), whereas the energy effect may be at least partly explained by the underconsumption of E− vs. E+ as shown during the first 15 min of distribution in previous experiments [32]. The fact that changes in atrogin-1 and MuRF1 expression were recorded at different times depending on the study (here and Ref. [10]), i.e., after two and seven cycles of sequential feeding and both 5 h and 24 h after switching diets, supports the hypothesis of some consistent mechanisms of atrogene regulation according to temporal changes in dietary provision, despite the dynamic nature of such regulation. In our experimental conditions, these changes in atrogene mRNA levels did not induce major alterations in chymotrypsin-like and trypsin-like activity measured at 5 h postswitch. A somewhat similar pattern between proteasomal peptidase activity and expression data was recorded, but the diet-related variations within a sequential feeding program were not so marked and failed to reach statistical significance, except for alternation of mixed diets. Although a relationship between atrogene expression and degradation capacity of the proteasome is not systematic, as reported in different models of muscle atrophy in trout for instance [28,33,34], we cannot exclude the possibility that more than 5 h is necessary after switching diet to obtain an enhanced effect.

In the present study, the use of three sequential feeding programs varying in either protein or energy, or both, also provides evidence of significant differences between atrogin-1 and MuRF1 regulation. In particular, the expression of atrogin-1 was almost twice as high in the

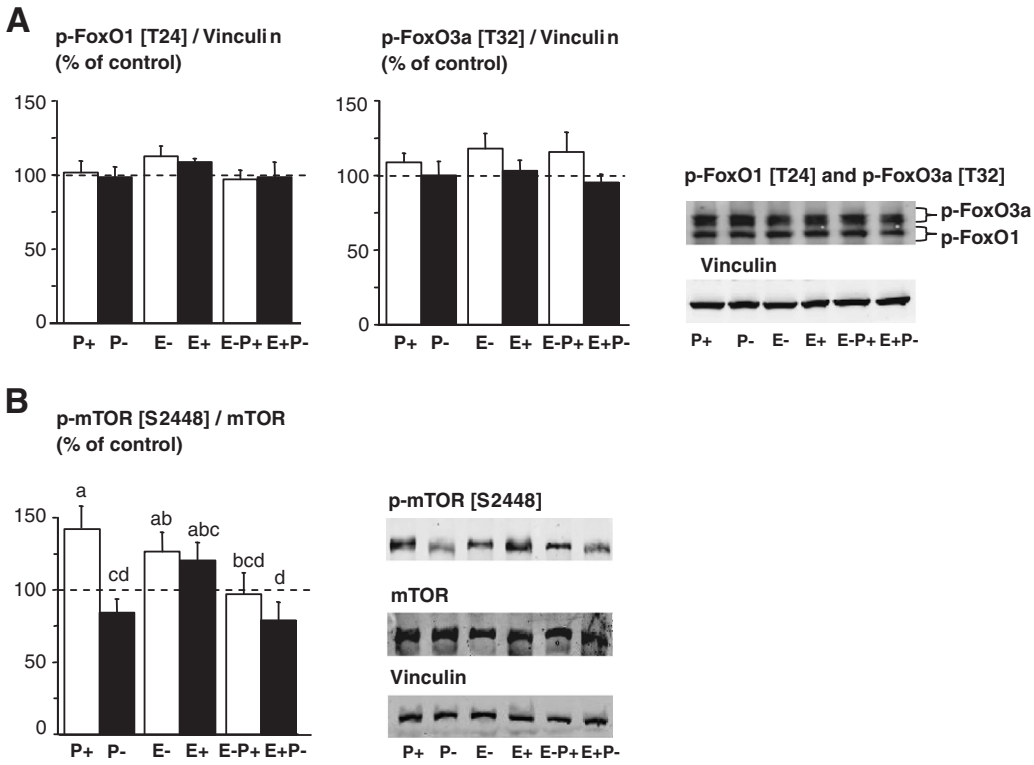

Fig. 2. Effects of daily changes in dietary protein and/or energy content on the phosphorylation of FoxOs (A) and mTOR (B) in breast muscle of 2-week-old chickens. Measurements were performed 5 h after the switch of diet. Representative Western blots performed using the antibodies indicated. Blots were quantified, and the p-FoxO1/vinculin, p-FoxO3a/vinculin and p-mTOR/mTOR ratios were determined. Results are expressed as percentages of the control on the day of the experiment and presented as means ± S.E.M. (n=6). Means not sharing the same letter are significantly different (P<.05).

E+P− diet than in the E−P+ diet, whereas that of MuRF1 was unchanged. These findings may be partly due to different changes induced by protein and energy provision according to the two E3 ubiquitin ligases. For MuRF1, the increase was approximately 1.7-fold in both P− compared to P+ and E− compared to E+ sequences, with potential compensation effects of energy and protein in the case of alternation of mixed diets. Conversely, for atrogin-1, the extent of stimulation was much more pronounced with cyclical changes in protein content (4.5-fold in P− vs. P+) compared to cyclical changes in energy content (2.7-fold in E− vs. E+), which is a possible explanation for the doubled atrogin-1 expression in the E+P− compared to the E−P+ diet. This particularly high effect of daily variations in protein levels specifically on atrogin-1 expression must be emphasized because it is very original. To our knowledge, the few studies in which changes in dietary provision have been tested on E3 ligases did not show such drastic and specific regulation of atrogin-1. A recent study indicated that atrogene expression may depend on the amino acid profile of dietary proteins, but without higher stimulation of atrogin-1 compared to MuRF1 [35]. The higher values were thus recorded with the zein protein (a very amino-acid-imbalanced protein with considerable lysine deficiency) that produced greatly delayed growth and, on the basis of previous experiments using lysine-deficient diets [36,37], probably induced changes in muscle protein breakdown.

In mammals and chickens, the transcription factors FoxOs are involved in the control of E3 ubiquitin ligases [10,14,15,38]. Here, FoxO1 phosphorylation was unchanged following daily variations in dietary protein and/or energy content, and variations in FoxO3a phosphorylation were not strong enough to be significant at 5 h postswitch. Regulation of atrogene expression with alternation of diets varying in energy independent of TOR signaling suggests that other mechanisms regulate atrogene expression that were not detectable in our experimental conditions (e.g., single collection at 5 h postswitch). The drastic increase in S6 phosphorylation on S235/S236 in the E+ diet compared to the E− diet without changes in the phosphorylation of TOR, S6K1 and 4EBP1, which is an interesting but unexplained response, also indicates the complexity of the underlying mechanisms. The signaling pathways that mediate control of gene transcription are only partially understood, and additional experiments are still needed to define the precise cascade of molecular events involved.

The kinase mTOR was clearly regulated by alternation of diets varying in protein (lower phosphorylation in P− vs. P+) and may contribute to modulation of atrogin-1 expression in these conditions (overexpression in P− vs. P+) since, as shown by *in vitro* studies [16,17,39], it is involved in the regulation of this atrogene. The changes observed in atrogin-1 expression and the mTOR cascade may originate from variations in anabolic factors such as amino acids. In particular, the major effect of daily variations in protein content, which led here to a significant increase in plasma amino acid concentrations, is consistent with findings demonstrating the control of this E3 ligase by amino acids *in vitro* [16,17,40,41]. The role of amino acids *in vivo* is less clear. For example, muscle atrogin-1 has been found to be unresponsive to leucine in rats in two experimental conditions, i.e., acutely and chronically elevated plasma leucine concentrations [42]. In our experimental conditions, it is possible that amino acids acted in synergy with insulin, whose plasma concentrations should have been transiently changed with differences between diets, although these differences were not significant at the time of sampling. Interestingly, we observed concomitant changes in atrogin-1 expression and in phosphorylation levels of mTOR targets involved in mRNA translation into proteins (e.g., lower phosphorylation of S6K1, S6 and 4EBP1 in P− vs. P+). These findings may corroborate recent evidence indicating that atrogin-1 is related

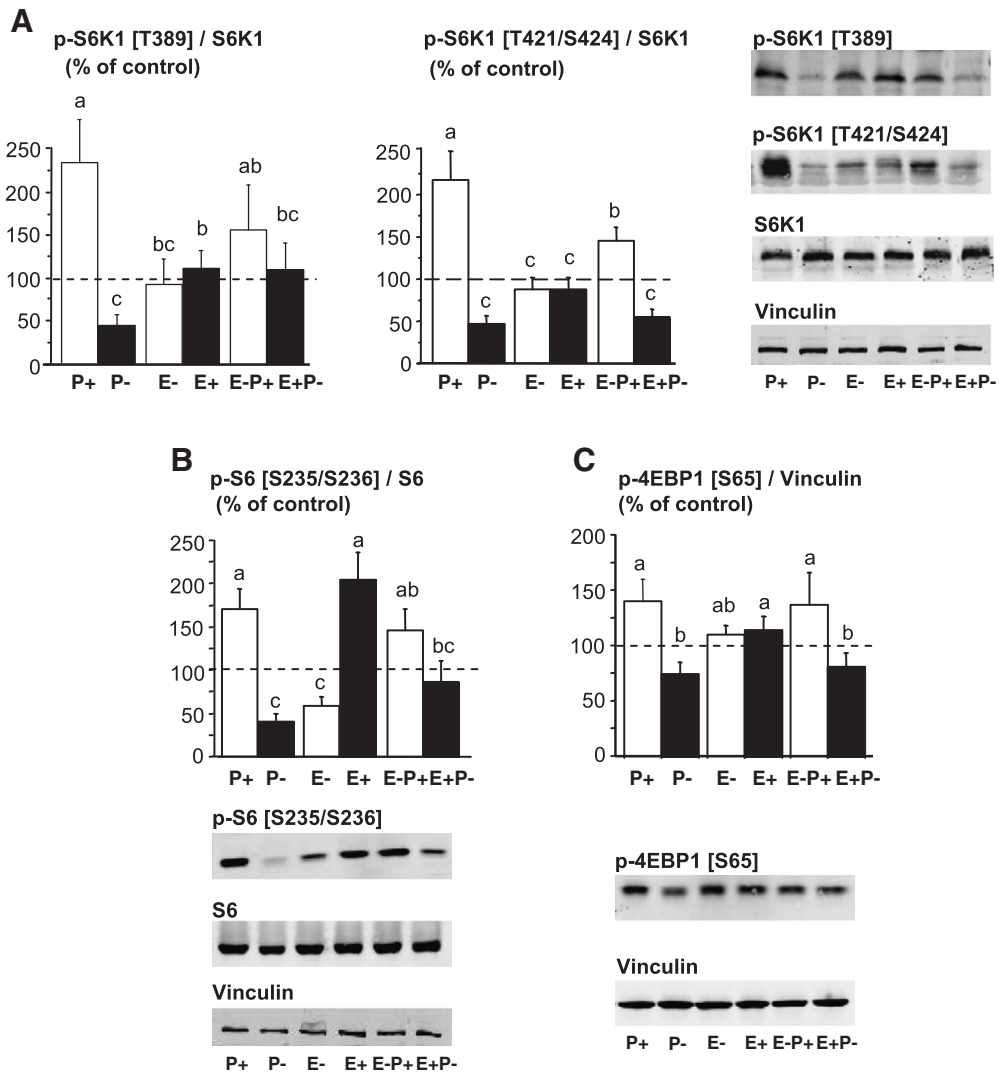

Fig. 3. Effects of daily changes in dietary protein and/or energy content on the phosphorylation of mTOR downstream targets related to translation initiation in breast muscle of 2-week-old chickens. Phosphorylation of S6K1 (A), S6 (B) and 4EBP1 (C). Measurements were performed 5 h after the switch of diet. Representative Western blots performed using the antibodies indicated. Blots were quantified, and the p-S6K1[T389]/S6K1, p-S6K1[T421/S424]/S6K1, p-S6[S235/S236]/S6 and p-4EBP1[S65]/vinculin ratios were determined. Results are expressed as percentages of the control on the day of the experiment and presented as means ± S.E.M. (n=6). Means not sharing the same letter are significantly different (P<.05).

to muscle protein synthesis and growth through the regulation of downstream effectors of mTOR, and that the activity or inactivity of a common set of molecules controlling specific cellular pathways determines whether the skeletal muscle tissue will respond to defined stimuli with muscle growth or loss [13,43,44]. eIF3f has been characterized as a substrate of atrogin-1 [45]. eIF3f plays a key role in muscle hypertrophy by increasing the activity of the TOR pathway, and the overexpression of an eIF3f mutant resistant to degradation is associated with protection against muscle atrophy [43,44]. The specific targeting of a particular protein (eIF3f) by atrogin-1 may explain the decreased protein synthesis observed in atrophy. It is noteworthy that although MuRF1 and atrogin-1 are E3 ubiquitin ligases, the specificity of their substrates means that they probably have different roles in the control of muscle mass. MuRF1 is involved in the degradation of myofibrillar proteins since myosin heavy chain and other myofibrillar proteins are targeted for breakdown by the proteasome in a MuRF1-dependent manner [11,12]. MuRF1 may thus be associated with muscle proteolysis, whereas changes in atrogin-1 appear not to be systematically correlated with the rates of muscle proteolysis.

In conclusion, expression of atrogin-1 and MuRF1 is regulated in the short term (i.e., 5 h after the diet change) by 48-h cycle sequential feeding programs with diets varying in protein and/or energy content. In our experimental model designed to distinguish the effects of protein and energy provision on the regulation of muscle metabolism in chickens with maintained growth performance, we show some atrogene-specific regulation, with a particularly pronounced effect of protein sequence on atrogin-1. Our results also suggest that the translational efficiency of protein synthesis is affected through the regulation of the mTOR cascade without any impairment of the synthesis machinery (i.e., capacity for protein synthesis). The mechanisms coordinating the short-term regulation of the protein synthesis/proteolysis balance by nutrients remain to be characterized. These findings also provide a basis for our understanding of metabolic adaptation to variations in dietary provision. Sequential feeding models are valuable tools for exploring chrononutrition with the aim of developing alternative nutritional strategies. Such strategies could be used for several purposes such as improving overall protein deposition and optimizing feed utilization.

Acknowledgments

The authors thank Florence Favreau (INRA, UE1295 PEAT, Nouzilly, France) and Anne-Marie Chagneau (INRA, UR83 Recherches Avicoles, Nouzilly, France) for animal care and experimental design management, and Magali Bergès, Estelle Cailleau-Audouin, Sabine Crochet and Hamza Mameri (INRA, UR83 Recherches Avicoles, Nouzilly, France) for their technical assistance. They are also grateful to Christiane Vachot (INRA, UR1067 Nutrition, Métabolisme, Aquaculture, St. Pée-sur-Nivelle, France) for peptidase activity measurements and to D. Raine (Surrey, UK) for editing the English language.

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