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Differential regulation of protein synthesis by skeletal muscle type in chickens

Takaoki Saneyasu*, Yuma Nakano, Nami Tsuchii, Ayana Kitashiro, Tatsuya Tsuchihashi, Sayaka Kimura, Kazuhisa Honda, Hiroshi Kamisoyama

Graduate School of Agricultural Science, Kobe University, Kobe, Japan

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ABSTRACT

In mammalian skeletal muscles, protein synthesis rates vary according to fiber types. We herein demonstrated differences in the regulatory mechanism underlying the protein synthesis in the pectoralis major (a glycolytic twitch muscle), adductor superficialis (an oxidative twitch muscle), and adductor profound (a tonic muscle) muscles of 14-day-old chickens. Under *ad libitum* feeding conditions, protein synthesis is significantly higher in the adductor superficialis muscle than in the pectoralis major muscle, suggesting that protein synthesis is up-regulated in oxidative muscles in chickens, similar to that in mammals. In the pectoralis major muscle, fasting significantly inhibited the Akt/S6 pathway and protein synthesis with a corresponding decrease in plasma insulin concentration. Conversely, the insulin like growth factor-1 (IGF-1) mRNA levels significantly increased. These findings suggest that the insulin/Akt/S6 pathway plays an important role in the regulation of protein synthesis in the pectoralis major muscle. Interestingly, protein synthesis in the adductor superficialis muscle appears to be regulated in an Akt-independent manner, because fasting significantly decreased S6 phosphorylation and protein synthesis without affecting Akt phosphorylation. In the adductor profound muscle, IGF-1 expression, phosphorylation of Akt and S6, and protein synthesis were decreased by fasting, suggesting that insulin and/or skeletal IGF-1 appear contribute to protein synthesis via the Akt/S6 pathway. These findings revealed the differential regulation of protein synthesis depending on skeletal muscle types in chickens.

1. Introduction

Skeletal muscles are the most abundant tissue in vertebrates, and its mass increases during postnatal development (Sandri, 2013; Schiaffino et al., 2013). Insulin and insulin like growth factor-1 (IGF-1) are well known regulators of skeletal muscle growth. They activate the Akt/mTOR/S6K1 signaling pathway and ultimately induce the phosphorylation of ribosomal protein S6 (S6) (Glasss 2005; Sandri, 2008, 2013; Schiaffino et al., 2013; Tesseraud et al., 2007). Therefore, insulin, IGF-1 expression, and Akt phosphorylation as well as S6 phosphorylation have been generally analyzed in research on protein synthesis in skeletal muscles.

Mammalian skeletal muscle fibers are classified into four fiber types based on the physiological and biochemical properties: slow-twitch oxidative (SO) type I, fast-twitch oxidative glycolytic (FOG) IIA, fast-twitch glycolytic (FG) IIB, and FG IIX/D. Protein synthesis rates and S6 and Akt phosphorylation are different between glycolytic and oxidative fibers (van Wessel et al., 2010). For example, the rate of Akt phosphorylation is higher in the rat soleus muscle (SOL, 13% by FOG and 87% by SO) than in the extensor digitorum longus (EDL, 42% by FOG, 56% by FG, and 2% by SO; Sakamoto et al., 2002; Armstrong and Phelps, 1984). Although the content of total p70S6K1 protein is higher in rat EDL than in the SOL (Atherton et al., 2004), the basal phosphorylation level is lower in the EDL than in the SOL (Hornberger et al., 2001). Furthermore, a novel *in vivo* technique revealed that type IIB and IIX fibers have significantly lower rates of protein synthesis than type IIA fibers in the mouse plantaris muscle (PLT, 21% by type IIA, 77.4% by type IIX and IIB, and 1.6% by type I; Agbulut et al., 2003) and SOL (31.2% by type IIA, 15.2% by type IIX, and 53.6% by type I; Agbulut et al., 2003); only small differences in protein synthesis rates exist between type IIA and type I fibers (Goodman et al., 2011).

In chickens, skeletal muscle fiber types have been histochemical classified as type I, IIA, IIB, IIIA, and IIIB (Barnard et al., 1982). Similar to mammals, type I and IIA fibers in chickens are oxidative twitch fibers, and type IIB fibers are glycolytic twitch fibers. Type III fibers are tonic fibers that are expressed in the anterior latissimus dorsi, adductor

* Corresponding author at: Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.

E-mail address: saneyasu@phoenix.kobe-u.ac.jp (T. Saneyasu).

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profound, and plantaris muscles in chickens. Type III fibers appear to be oxidative fibers based on their histochemical properties (Barnard et al., 1982) and their lipid metabolism-related genes expression patterns (Saneyasu et al., 2015). Previous studies in newly hatched chicks have showed that the levels of IGF-1 mRNA and phosphorylated Akt protein were significantly higher in pectoralis major muscles, which predominantly consists of IIB fibers, than in sartorius muscles, which consists of type I, IIA, and IIB fibers (Barnard et al., 1982; Ijiri et al., 2014; Shimamoto et al., 2016). However, whether protein synthesis and its regulatory mechanisms differ between muscle fiber types in chicken skeletal muscle has not yet been elucidated. Therefore, we compared protein synthesis and the expression levels of protein synthesis-related factors in three different types of muscles (pectoralis major, adductor superficialis, and adductor profound muscle) in chickens under feeding and fasting conditions.

2. Materials and methods

2.1. Animals and feeding

Animal experiments in the present study were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulation.

Day-old male chickens (Ross308) were purchased from local hatcheries (Ishii Poultry Farming Cooperative Association, Tokushima, Japan). They were given free access to water and a commercial chicken starter diet (23.5% crude protein and 3050 kcal/kg, Nippon Formula Feed Mfg. Co. Ltd., Kanagawa, Japan).

2.2. Sampling and preparation

2.2.1. Measurement of protein synthesis

Protein synthesis was measured as described in the previous study (Goodman et al, 2011; Saneyasu et al., 2017b). Briefly, 13-day-old chickens were allocated to two groups based on body weight (five birds in each group). One group was given food and water *ad libitum*, and the other group was deprived of food for 24 h. The body weights were measured, and the chicks were intravenously injected with puromycin (20 nmol/g body), a structural analog of tyrosyl-tRNA. It has been demonstrated that when puromycin is used at low concentrations, the accumulation of puromycin-conjugated peptides accurately reflects the rate of protein synthesis (Goodman et al, 2011). Exactly 15 min after the injection, the chicks were euthanized by decapitation, and the pectoralis major, adductor superficialis, and adductor profound muscle, which almost completely consists of type IIB fibers (> 99%), primarily of type IIA fibers (80–95%), and completely of type III fibers (100%), respectively (Barnard et al., 1982), were excised in that order. The dissected muscles were immediately frozen in liquid nitrogen and stored at −80 °C for western blot analysis to detect the puromycin-labelled peptides.

2.2.2. Effects of fasting on levels of protein synthesis-related factors

Twelve 13-day-old chickens were allocated based on body weight to two groups of six birds. One group was given food and water *ad libitum*, and the other group was deprived of food for 24 h. Next, chickens were euthanized by decapitation. Blood was collected from the carotid artery. Plasma was separated immediately by centrifugation at 3000 g for 10 min at 4 °C, and the plasma insulin concentration was measured using a commercial kit (Rat Insulin ELISA KIT (TMB), Shibayagi, Gunma, Japan) as described in a previous study (Saneyasu et al., 2017b). The skeletal muscles were excised. The dissected muscles were immediately frozen by liquid nitrogen and stored at −80 °C for further analysis.

Table 1
Primer sequences used for real-time PCR analysis.

Gene name	Forward primer	Reverse primer	Accession number
IGF-1	5′-gct gcc ggc cca gaa-3′	5′-acg aac tga aga gca tca acc a-3′	NM_001004384
RPS17	5′-gcg ggt gat cat cga gaa gt-3′	5′-gcg ctt gtt ggt gtg aag t-3′	NM_204217

IGF-1, insulin-like growth factor-1; RPS17, ribosomal protein S17. The primers were used in previous study (Saneyasu et al., 2016, 2017a; 2017b).

2.3. Real-time PCR analysis

Real-time PCR analysis was performed as described in the previous study (Saneyasu et al., 2016, 2017a, 2017b). Briefly, total RNA was extracted from the muscle using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) according to supplier's instruction. First-strand cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Mater Mix with gDNA Remover (Toyobo Co. Ltd, Osaka, Japan) according to the supplier's instruction. mRNA levels of IGF-1 and ribosomal protein S17 (RPS17) were analyzed by relative standard curve method using the Thermo Scientific PikoReal Real-Time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland), each primer (Table 1), and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The efficiency of the PCR amplification was determined by the standard dilution curve. Melting curve analysis showed a single peak for both primer pairs. The amplicons were sequenced to confirm their specificity. Expression levels of IGF-1 were normalized to those of RPS17, because we previously confirmed that RPS17 was better suited as the reference gene when compared to 18S ribosomal RNA and GAPDH in an analysis on different skeletal muscles using Normfinder, which identifies the optimal normalization genes (Saneyasu et al., 2015).

2.4. Western blot analysis

Western blot analysis was performed as described in the previous study (Saneyasu et al., 2017b). Briefly, frozen muscles samples were ultrasonicated in a lysis buffer containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 0.5% NP-40, 100 mM sodium fluoride, 23 mM sodium phosphate, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Homogenates were centrifuged at 17,900 × *g* for 15 min at 4 °C, and supernatants were stored at −80 °C. Protein concentration were determined by Lowry's method (Lowry et al., 1951). Muscle lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting using the HorizeBlot (ATTO Co., Tokyo, Japan) according to the supplier's recommendations. Bands were measured by Chemi-Lumi one Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with the Lumicube (Liponics Inc., Tokyo, Japan), and quantified using CS Analyzer (ATTO Co., Tokyo, Japan). Anti-Akt (#9272), anti-p-Akt (Ser473) (#9271), anti-S6 (#2217), anti-p-S6 (Ser240/244) (#5364), anti-β-actin (#4967), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). The species cross-reactivity is described in the manufacture's data sheet. Anti-puromycin (MABE343) and HRP-conjugated anit-mouse IgG (sc-2005) were purchased from EMD Millipore (Temecula, CA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Anti-β-actin was used as a loading control.

2.5. Statistical analysis

All statistical analyses were performed using Excel 2013 (Microsoft,

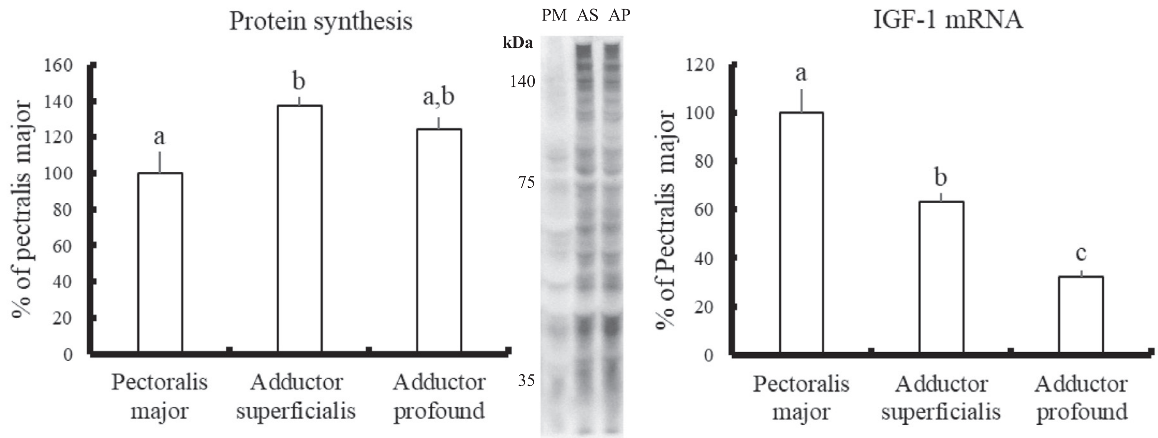

Fig. 1. Comparison of protein synthesis and IGF-1 expression between different types of skeletal muscle in broiler chickens. Data are presented as mean ± SEM (protein synthesis, n = 5; IGF-1 mRNA, n = 6). The Tukey-Kramer method was performed to analyze differences. Groups with different letters were significantly different ($P < 0.05$). PM, pectoralis major; AS, adductor superficialis; AP, adductor profound.

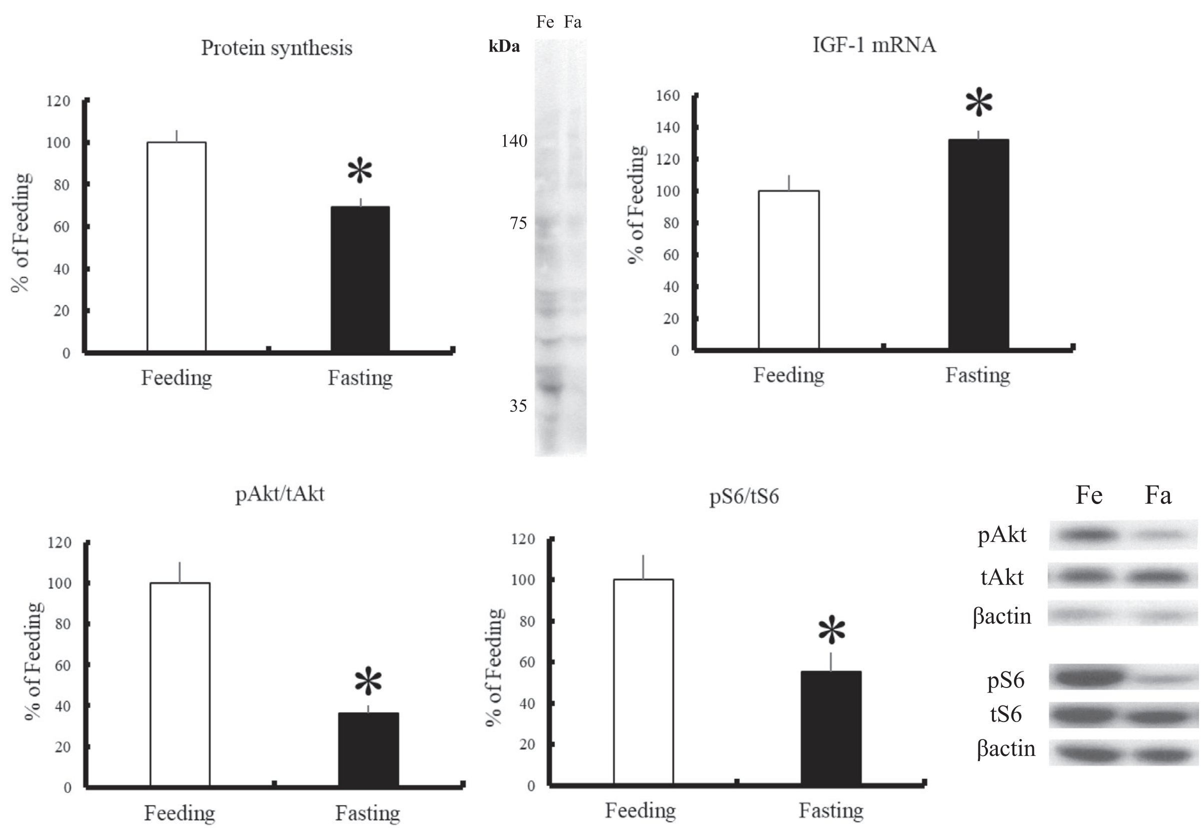

Fig. 2. Effects of fasting on protein synthesis, IGF-1 expression, and phosphorylation of Akt and S6 in pectoralis major muscle of broiler chicks. Data are means ± SEM (n = 5 or 6). Differences were analyzed by Student's *t*-test. *, significantly different from the feeding group within each type of muscle ($P < 0.05$). Fe, feeding; Fa, fasting.

USA) with the add-in software Statcel 3 (OMS, Tokyo, Japan). Data on protein synthesis and IGF-1 expression levels under feeding conditions were analyzed using the Tukey-Kramer method. The Student's *t*-test was performed to analyze differences in plasma insulin concentration, mRNA/protein levels, and protein synthesis between feeding and fasting conditions.

3. Results

Under feeding conditions, protein synthesis was significantly ($P < 0.05$) higher in the adductor superficialis muscle than in the pectoralis major muscle (Fig. 1, Suppl. Fig. 1), suggesting protein synthesis is more upregulated in chicken oxidative fibers than in chicken glycolytic fibers. In contrast, IGF-1 mRNA levels in the adductor superficialis muscle were significantly ($P < 0.05$) lower than in the pectoralis major muscle (Fig. 1). Additionally, although no significant difference was observed in protein synthesis in the adductor profound muscle when compared to other muscles, the IGF-1 mRNA levels were significantly lower in the adductor profound muscle than in other muscles (Fig. 1, Suppl. Fig. 1). Therefore, skeletal IGF-1 may not

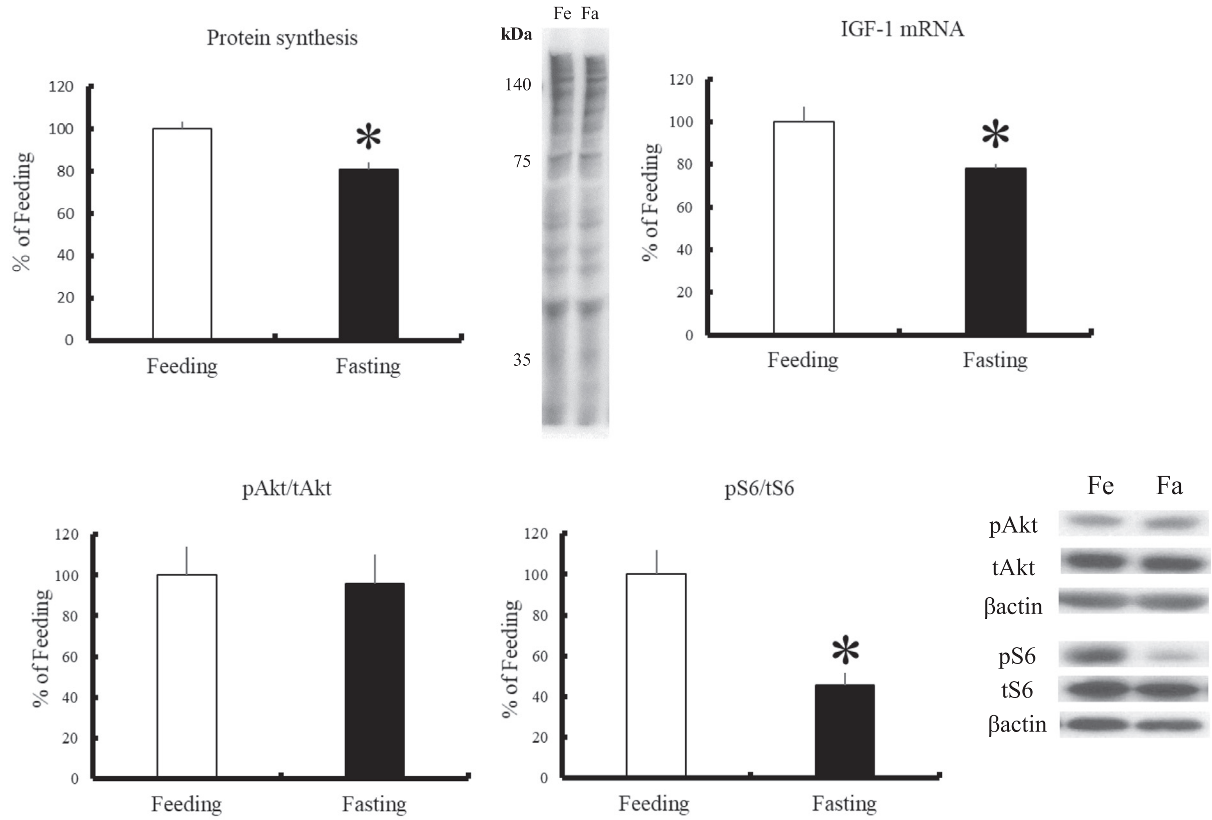

Fig. 3. Effects of fasting on protein synthesis, IGF-1 expression, and phosphorylation of Akt and S6 in adductor superficialis muscle of broiler chicks. Data are mean ± SEM (n = 5 or 6). Differences were analyzed by Student's *t*-test. *, significantly different from the feeding group within each type of muscle ($P < 0.05$). Fe, feeding; Fa, fasting.

cause the difference in protein synthesis between the skeletal muscle types.

Alphaβ-tubulin and β-actin protein are generally used as a loading control in Western blot analysis. Unexpectedly, a preliminary study showed significant ($P < 0.05$) differences in the band intensity of these loading controls between the three muscles (Suppl. Fig. 2). Therefore, it is likely inappropriate to compare protein levels between the three muscles in the present study via Western blot analysis.

Fasting significantly ($P < 0.01$) decreased the plasma concentration of insulin (feeding, 2.86 ± 0.40 ng/mL; fasting, 1.09 ± 0.15 ng/mL).

In the pectoralis major muscle, fasting significantly ($P < 0.05$) inhibited protein synthesis (Fig. 2, Suppl. Fig. 1). Similarly, the phosphorylation of Akt and S6 was significantly ($P < 0.05$) decreased by fasting (Fig. 2). In contrast, fasting significantly ($P < 0.05$) increased IGF-1 mRNA levels (Fig. 2). Therefore, skeletal IGF-1 may not be involved in the fasting-induced downregulation in protein synthesis or Akt/S6 pathway in the chicken pectoralis major muscle.

Interestingly, although fasting significantly ($P < 0.05$) inhibited protein synthesis (Fig. 3, Suppl. Fig. 1), IGF-1 expression, and S6 phosphorylation in the adductor superficialis muscle, no significant change was observed in Akt phosphorylation (Fig. 3). It is therefore likely that protein synthesis is regulated in an Akt-independent manner in chicken adductor superficialis muscle.

Similar to the results in the pectoralis and adductor superficialis muscle, protein synthesis and S6 phosphorylation were significantly ($P < 0.05$) downregulated in the fasted state in the adductor profound muscle (Fig. 4, Suppl. Fig. 1). Additionally, IGF-1 expression significantly ($P < 0.05$) decreased and Akt phosphorylation decreased, albeit not significantly, ($P = 0.08$) under the fasting conditions (Fig. 4). These results suggest that insulin and/or skeletal IGF-1 regulates protein synthesis via the Akt/S6 pathway in chicken adductor profound muscle.

4. Discussion

In murine skeletal muscles, type IIB and IIX fibers have significantly lower rates of protein synthesis than type IIA fibers; only small differences in protein synthesis rates exist between type IIA and type I fibers (Goodman et al., 2011). Similarly, previous studies in carp reported that the protein synthesis rate was higher in red muscles than in white muscles (Smith et al., 1996; Watt et al., 1988). The present result is similar to the previous studies; protein synthesis was more upregulated under feeding conditions in the adductor superficialis muscle than in the pectoralis major muscle. Therefore, all these findings demonstrate a common phenomenon; in vertebrates, protein synthesis is more active in oxidative muscle than in glycolytic muscle.

Unlike the protein synthesis results in this study which showed higher synthesis in the adductor superficialis muscle than in the pectoralis major muscle, the IGF-1 mRNA levels were significantly lower in the adductor superficialis and profound muscle than in the pectoralis major muscle under feeding conditions. This discrepancy might be caused by a difference in total RNA content between muscle fiber types. Total RNA per microgram of muscle tissue is 2.3-fold higher in rat SOL compared to EDL (van Wessel et al., 2010), and several genes have higher expression in SOL than in EDL when normalized by microgram muscle tissue (van Wessel et al., 2010). Additionally, since ribosomal RNA content reflects the number of ribosomes, the higher total RNA content in rat SOL compared to EDL implies that high oxidative fibers have a higher capacity to synthesis protein (van Wessel et al., 2010). It is therefore likely that the total RNA content and protein synthesis capacity are higher in the oxidative adductor superficialis and profound

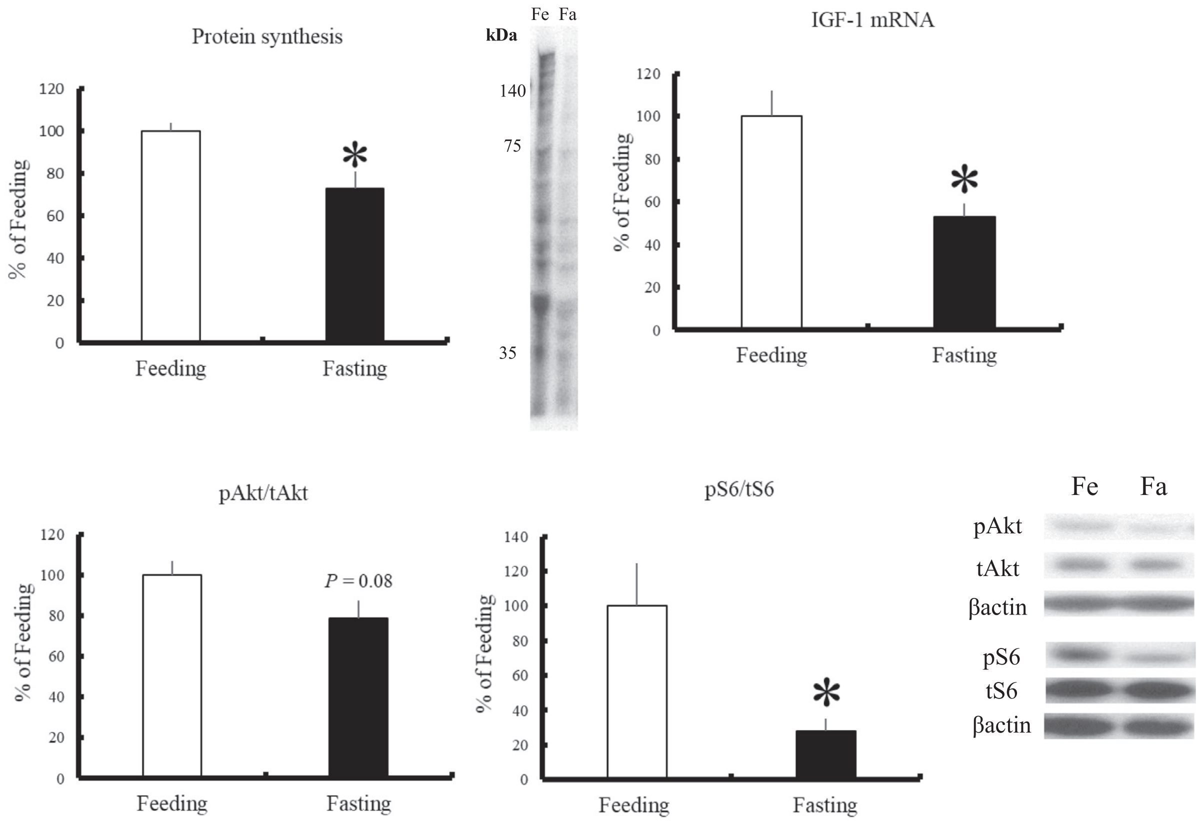

Fig. 4. Effects of fasting on protein synthesis, IGF-1 expression, and phosphorylation of Akt and S6 in adductor profound muscle of broiler chicks. Data are presented as means ± SEM (n = 5 or 6). Differences were analyzed by Student's *t*-test. *, significantly different from the feeding group within each type of muscle ($P < 0.05$). Fe, feeding; Fa, fasting.

muscle than in the glycolytic pectoralis major muscle; this may be the reason why protein synthesis is significantly upregulated in the adductor superficialis muscle compared to the pectoralis major muscle, even though IGF-1 mRNA levels normalized by RPS17 are lower in the adductor superficialis muscle.

A previous study in broiler chickens showed that short-term fasting significantly inhibited phosphorylation of Akt and S6 in the pectoralis major muscle with a corresponding decrease in plasma insulin concentration, whereas no significant change was observed in the IGF-1 mRNA levels in pectoralis major muscle (Saneyasu et al., 2017b). Similarly, the present study showed that the Akt/S6 pathway and protein synthesis in the pectoralis major muscle was downregulated by long-term fasting with a corresponding decrease in plasma insulin concentration, but no decreases in skeletal IGF-1 mRNA levels. In contrast, refeeding and insulin injection significantly promotes phosphorylation of Akt, mTOR, S6K1, and S6 in pectoralis major muscles of broiler chickens (Duchêne et al., 2008). All these findings suggest that insulin is a main regulator of the Akt/S6 pathway and protein synthesis in response to nutritional conditions in chicken pectoralis major muscles.

Although IGF-1 expression was not inhibited in the pectoralis major muscle by fasting in the present and previous studies, other previous studies in chickens with different growth rates suggest that skeletal IGF-1 plays an important role in skeletal muscle growth (Jia et al., 2018; Xiao et al., 2017). We previously reported that IGF-1 mRNA levels in pectoralis major muscles increased under feeding conditions in an age-dependent manner during the first week after hatching, in association with the changes in phosphorylation rate of Akt and S6 (Saneyasu et al., 2017a). IGF-1 expression and Akt phosphorylation drastically decreased 2 weeks after hatching (Saneyasu et al., 2016). Therefore, it is possibly that skeletal IGF-1 determines the basal level of the Akt/S6 pathway and growth rate in chicken pectoralis major muscles.

Adductor superficialis muscles primarily consist of oxidative type IIA fibers (Barnard et al. 1982). Interestingly, the present study showed that fasting failed to inhibit Akt phosphorylation in the adductor superficialis muscle, in contrast to the other muscles. A previous study in rats reported that phospho-Akt expression was significantly decreased by 1 day-fasting in PLT, whereas it was significantly decreased by 2 day- but not 1 day-fasting in SOL (Ogata et al., 2010). It is therefore likely that Akt phosphorylation in oxidative fibers responds more slowly to nutritional conditions when compared to that in glycolytic fibers. However, it remains unclear how protein synthesis is regulated in an Akt-independent manner in the adductor superficialis muscle. One of the possible mechanisms is leucine regulation; fasting significantly decreases leucine content in rat SOL (Holeček and Mičuda 2017) and oral administration of leucine significantly increases phosphorylation of S6K1 and S6 but not Akt in rat skeletal muscle (Le Plénier et al., 2012). Further studies are required to clarify the regulatory mechanisms of protein synthesis in chicken oxidative fibers.

The adductor profound muscle is known to be a tonic muscle and completely consists of type III fibers (Barnard et al., 1982). Several studies suggest that type III fibers are oxidative fibers in chicken skeletal muscle (Barnard et al., 1982; Saneyasu et al., 2015; Skiba-Cassy et al. 2007). In the present study, insulin and/or skeletal IGF-1 appears to regulate protein synthesis in the adductor profound muscle. Unlike in the adductor superficialis muscle, Akt phosphorylation in the adductor profound muscle is likely involved in the regulation of protein synthesis. It is therefore likely that the regulatory mechanism of protein synthesis in type III fibers differs from that in oxidative type IIA fibers.

In the present study, we analyzed Akt phosphorylation on Ser473. This phosphorylation is catalyzed by mTORC2 and required for maximal activation of Akt (Dibble and Cantley, 2015). Therefore, the present results indicate that fasting may affect mTORC2 activity in a manner that varies between muscle fiber types in chickens. Although there is evidence that mTORC2 activity is stimulated in a phosphoinositide 3-kinase-depenent manner, the underlying mechanism is incompletely understood (Dibble and Cantley, 2015). Further comparative studies between different types of muscle may help elucidate the mechanisms regulating mTORC2 activity.

The pectoralis major muscle, which constitutes the largest part of the muscles of the shoulder girdle, is used to lower the wing. In contrast, the adductor superficialis and profound muscle, which constitute part of thigh muscles, are postural muscles. Therefore, there is a possibility that the present result is caused by differences in location and motor task of the skeletal muscles analyzed. Further comparative analyses involving the same skeletal muscle may be required.

5. Conclusion

We compared protein synthesis and the expression of protein synthesis-related factors in different types of muscles in chickens under feeding and fasting conditions. The results suggest that protein synthesis is lower in glycolytic twitch muscles than in oxidative twitch muscles, and that the regulatory mechanisms of protein synthesis differs between oxidative and glycolytic twitch muscles in chickens.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113246.

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