Refeeding and Insulin Regulate S6K1 Activity in Chicken Skeletal Muscles

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ABSTRACT Broiler chickens are characterized by fast muscle growth and high protein deposition, most likely subsequent to a high protein synthesis. However, the regulation of protein synthesis in chicken muscle is still unknown. In contrast, it has been clearly demonstrated in mammals that S6K1 is a key regulator of protein synthesis. In the present study, S6K1 was characterized in both pectorals and gastrocnemius muscles in chickens. A 133-bp fragment of chicken S6K1 cDNA had 84% identity to mammalian S6K1. We investigated in vivo the effects of refeeding and insulin treatment after 16 h starvation. S6K1 enzyme activity was significantly increased in both pectorals and gastrocnemius muscles by refeeding (two- to threefold greater than in food-deprived chickens, P < 0.05). Optimal activation occurred 30 min after refeeding following 16 h starvation. S6K1 activation was associated with its phosphorylation on serine and Thr 389 residues, which occurred within the first 5 min of refeeding. S6K1 was also significantly stimulated in both pectorals and gastrocnemius muscles after a single insulin injection (nine- to 12-fold greater than in control chickens, P < 0.001). Our results indicate that S6K1 is expressed in chickens muscles and activated by refeeding and insulin treatment. J. Nutr. 133: 369–373, 2003.

KEY WORDS: • protein synthesis • skeletal muscle • phosphorylation • chickens

S6K1,4 a cytoplasmic serine/threonine kinase, is critical for translational regulation of genes that encode essential components of the protein synthesis apparatus (1). S6K1 belongs to the ribosomal S6 kinase family, which also includes p90 S6 kinase (p90rsk). Both S6K1 and p90rsk can phosphorylate the ribosomal protein S6 in vitro but S6K1 is probably responsible for in vivo phosphorylation of S6 (2,3). Numerous studies have reported that S6K1 plays a key role in the control of insulin-dependent protein synthesis (4,5). After insulin receptor autophosphorylation and phosphorylation of insulin receptor substrate (IRS) proteins, the phosphatidylinositol 3-kinase (PI 3-kinase) pathway is activated and protein kinase B (PKB), the mammalian target of rapamycin/FKBP rapamycin-associated protein (mTOR/FRAP), PHAS-1 and S6K1 are each phosphorylated (4,5). Protein synthesis is also dependent on nutritional status; in rats and mice, food intake activates S6K1 and protein synthesis (6). Among nutrients, amino acids, and particularly branched-chain amino acids, are considered to be major activators of S6K1 (7,8).

Recent studies have shown that supplying amino acids after a period of amino acid deprivation can stimulate the activity of S6K1 (9–11). The contribution of nutrients and/or insulin to the stimulation of protein synthesis by refeeding is only beginning to be elucidated. There is in vitro and in vivo evidence of a signaling role of amino acids to increase protein synthesis by phosphorylating PHAS-1 and S6K1 (7,12). Refeeding type 1 diabetic mice increases protein synthesis in vivo despite their insulin resistance (6). Furthermore, refeeding rats a protein-deficient diet does not activate protein synthesis, indicating that insulin alone is not sufficient (13). These findings suggest that both amino acids and insulin activate protein synthesis and S6K1 through different pathways. It has in fact been demonstrated in vitro that S6K1 activation by amino acids is mediated by the mTOR pathway (12,14), whereas its activation by insulin involves the PI 3'-kinase pathway (4,15). However, other studies have shown that insulin can also activate S6K1 through a PI 3'-kinase-independent pathway (8). Thus, the precise mechanism by which insulin and amino acids activate S6K1 remains unclear.

The regulation of S6K1 is poorly documented in nonmammalian species. Therefore, we studied the mechanism of regulation of S6K1 in chickens. Chickens have high plasma levels of glucose (11 mmol/L) and a relative resistance to exogenous insulin (16). It is noteworthy that chickens have high growth and muscle protein synthesis rates (17). This animal model could therefore be very useful to understand the regulation of S6K1 in muscle, depending on nutritional or hormonal status in vivo. We previously demonstrated that the early steps of insulin signaling are present in chicken liver and muscle (18,19). In the liver, the insulin signaling cascade is highly sensitive to nutritional state; refeeding starved chickens leads to the phosphorylation of IR (insulin receptor), IRS-1 (insulin receptor substrate 1) and Shc (Src homology and collagen protein) and to the activation of PI 3'-kinase. Interestingly, despite the presence of all components of insulin signaling,
chicken muscle seems to be refractory to nutritional status except for the activation of Shc phosphorylation (20). These findings contrast with the fast growth of chicken muscle and the potential role of insulin in muscle protein synthesis. To address this question, we characterized chicken S6K1 and studied its expression in two muscle types: pectoralis major (glycolytic muscle type) and gastrocnemius (oxidative and glycolytic muscle type).

MATERIALS AND METHODS

Chemicals. BSA (fraction V, radioimmunoassay grade), leupeptin, aprotinin and protein A-agarose were purchased from Sigma Chemical (St. Louis, MO). RNA Insta-Pure system kit and TOPO TA cloning kit were provided by Invitrogen (Cergy-Pontoise, France), respectively. Nicotellulose membrane and S6K1 assay kit were purchased from Upstate Biotechnology (Euromedex, Mundolsheim, France) and premade polycrylamide solution Protogel was from Prolabo (National Diagnostic, Fontenay-sous-Bois, France). Anti-carboxyl terminal S6K1 (aS6K1) and anti-phospho S6K1 (Thr 389) were from Santa Cruz (Tebu, Le Perray-en-Yvelines, France) and anti-phosphoserine (aPser) from Chemicon International (Euromedex, Mundolsheim, France). [3P]ATP was obtained from NEN Life Sciences (Zaventem, Belgium).

Animals and experimental procedures. Male chickens (broiler poults) were housed in individual cages in a conventional temperature-controlled room, exposed to a daily 14-h light period. They consumed a balanced starter diet (12.12 MJ metabolized energy/kg containing 22% crude protein) and water ad libitum for 3 wk before the experiments. Three experiments were carried out on 3-wk-old chicks. After blood sampling, chickens were killed and muscles (pectoralis major muscle in expts. 1, 2 and 3 and gastrocnemius muscle in exp. 3) were removed, quickly frozen, powdered in liquid nitrogen and stored at −80°C.

Experiment 1. Chickens were separated into four groups and assigned to the following nutritional states: 1) food-deprived for 16 h (n = 9); 2) refed for 30 min after 16 h of food deprivation (n = 8); 3) refed for 120 min after 16 h food deprivation (n = 8) and 4) freely fed (n = 9).

Experiment 2. After 16 h of food deprivation, chickens were refed for 30 min. Blood and tissues were removed 1 to 270 min after the start of the refeeding period. Groups were food deprived for 16 h (n = 3), freely fed (n = 3) and kinetics of feed removal after the start of refeeding period were: 1 min (n = 3), 5 min (n = 3), 15 min (n = 3), 30 min (n = 3), 45 min (n = 3), 60 min (n = 3), 75 min (n = 3), 90 min (n = 3) and 150 min (n = 3).

Experiment 3. After 16 h of food deprivation, chickens were refed for 30 min (n = 3) or intravenously injected with insulin (1 U of insulin/kg) [insulin treatment 7 min (n = 1) and 15 min (n = 5)].

Determination of plasma glucose and insulin levels. Plasma glucose levels were measured by the glucose oxidase method using an automated analyzer (Glucose Beckman Analyzer 2; Palo Alto, CA). Plasma insulin levels were determined by radioimmunoassay with a guinea pig anti-porcine insulin antibody using chicken insulin as the standard (21).

Partial S6K1 cloning and sequencing. Total RNA was extracted using RNA Insta-Pure system kit from 100 μg of muscle samples according to the manufacturer’s recommendations. RNA (1 μg) was reverse transcribed using avian-myeloblastosis-virus transcriptase in the presence of oligo-dT primers as previously described (19). The resulting cDNAs were subjected to PCR in the presence of Taq polymerase and primers chosen from the partial S6K1 sequence determined from a chicken cDNA library cloned in MDV-infected T-cells with GenBank accession number gi:6579378 (sense primer 5′-TGA CCT TGG CAT GGA ACA TT-3′ and antisense primer 5′-TAC CTT TCC AAT GCC ACC TT-3′). The amplified fragments were separated on agarose gel (1%) and a band with the expected molecular weight (130 bp) was purified. The fragment was cloned using the TOPO TA cloning kit and automatically sequenced by Genome express (Paris, France).

Western blot analysis. Protein samples were prepared as previously described (18). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Muscle lysates (20 μg of protein) were subjected to SDS–PAGE gel electrophoresis and Western blotting using an anti-S6K1 antibody. Bands were revealed by enhanced chemiluminescence (ECL) after the action of horseradish peroxidase (HRP)–linked anti-rabbit γ-globulin. In some cases, solubilized materials (500 μg or 1 mg of protein) were subjected to immunoprecipitation using anti-S6K1 (final dilution of 1:50) or anti-phospho S6K1 (Thr 389) (final dilution 1:50) antibodies. The immune complexes were precipitated by the addition of protein–A–agarose beads for 2 h at 4°C. Immunoprecipitated proteins were subjected to SDS–PAGE gel electrophoresis and Western blotting using the appropriate antibodies. The activation of S6K1 in mammals is characterized by its phosphorylation on serine and threonine residues, inducing a gel retardation or shift. The phosphorylated active form of the kinase is determined as a band with lower mobility on SDS–PAGE and the unphosphorylated form, which corresponds to the inactive form, with a band with higher mobility.

S6K1 assay. S6K1 activity was measured in muscle extracts by immunoprecipitation to SDS–PAGE gel electrophoresis in the S6 kinase assay kit (Euromedex, Mundolsheim, France). Briefly, muscle homogenates were immunoprecipitated with anti-S6K1 antibody and the specific enzyme activity of the protein was measured by estimating the phosphorylation of an artificial substrate (AKRRRLSSRA) corresponding to an 11 amino acid sequence of the ribosomal protein S6 in the presence of labeled ATP.

Statistical analysis. Statistical analysis was performed using ANOVA (Statview Software program, version 5; SAS Institute, Cary, NC) to detect significant intergroup differences. Values are expressed as means ± SEM, and P < 0.05 was considered statistically significant. Comparisons were made between food-deprived chickens (control) and various groups using appropriate tests: Student–Newman–Keuls test was performed to compare glycemia and insulinemia; Fisher test was performed to compare S6K1 activity (expts. 1 and 2); and S6K1 activity measured in exp. 3 was analyzed using a two-way ANOVA to determine the statistical significance of the main effects of treatment (refeeding and insulin injection) and muscle type.

RESULTS

Glucose and insulin levels. Body weights of food-deprived, freely fed and refed chickens did not differ (expt. 1, data not shown). In addition, the feed intake during the refeeding period was measured and chickens ingested 30% and 40% of the mean daily feed intake within 30 and 120 min of refeeding, respectively (data not shown). Glycemia was increased in fed (12.7 ± 0.16 mmol/L, P < 0.05) and refed (14.78 ± 0.55 mmol/L, P < 0.001) chickens compared to food-deprived chickens (10.50 ± 0.28 mmol/L). Plasma insulin concentration reached its maximum level after 30 min of refeeding, with about a 60-fold increase (1988 ± 315 pmol/L, P < 0.001) compared to food-deprived chickens (31.50 ± 5.25 pmol/L), then declined after 120 min of refeeding to reach the insulin plasma concentration of fed chickens (255.5 ± 43.75 pmol/L, P < 0.05 compared to food-deprived chickens). Plasma glucose increased after 15 min of refeeding (Fig. 1A, exp. 2) and then glycemia plateaued between 30 and 75 min before declining to the glycemia concentration of fed chickens. Insulin plasma concentrations increased from the first min of refeeding to reach a peak after 30 min before declining (Fig. 1B).

Cloning and sequencing of a fragment of chicken muscle homologous of S6K1 cDNA. Using primers chosen from the GenBank sequence (gi:6579378) and flanking a region of 130 bp, RT–PCR performed on RNA of both pectoralis major and gastrocnemius muscles showed fragments with the expected molecular weight (Fig. 2A). After cloning, the amplified fragments were sequenced and the sequence analysis showed 84% identity to mammalian S6K1 (human, rabbit and rat). This indicates that chicken muscle expresses the homolog of S6K1.
Characterization of S6K1 in chicken muscle. A band was detected at the expected molecular weight of about 70 kDa in pectoralis major muscle with an electrophoretic migration similar to mammalian S6K1 (lysates from human A431 cells; Fig. 2B, expt. 1). In addition, the band intensity was dependent on the amount of protein loaded. The same band was detected in gastrocnemius muscle but the level of expression was higher than that in pectoralis major muscle (Fig. 2C, expt. 3).

Impact of nutritional status on S6K1 activation. In the pectoralis major muscle, the S6K1 band was shifted after 30 and 120 min of refeeding, indicating phosphorylation and activation of the enzyme (Fig. 3A, expt. 1). Muscle homogenates from fed and food-deprived chickens were used as positive and negative controls, respectively. Interestingly, the shift mobility after 30 and 120 min of refeeding was similar to the shift in the fed state. In the gastrocnemius muscle, the S6K1 band was also shifted after 30 min of refeeding, as in the pectoralis major muscle (Fig. 3C, expt. 3). There was a higher level of protein expression in gastrocnemius muscle, as previously indicated in Figure 2C. The S6K1 band was shifted after 30 min of refeeding, whereas there was no shift between 1 and 5 min of refeeding (Fig. 3B, expt. 2). In addition, the gel retardation of S6K1 was maintained at the level of the shift in fed chickens for at least 120 min after food withdrawal.

S6K1 activity in pectoralis major muscle remained at the basal level during the first 5 min of refeeding and significantly increased (by about 1.7-fold) at 30 min of refeeding compared to the enzyme activity determined in food-deprived chickens (Fig. 3B, expts. 1 and 2). S6K1 activity remained higher after 120 min of refeeding compared to that in food-deprived chickens, whereas when food was withdrawn after 30 min of refeeding (time, 150 min) S6K1 activity progressively decreased to values not different from those in the fed chickens. The enzyme activity in fed and food-deprived chickens did not differ. As in the pectoralis major muscle, 30 min of refeeding increased S6K1 activity in the gastrocnemius muscle (Fig. 3C, expt. 3). S6K1 activity was about three- and twofold greater in gastrocnemius and pectoralis major muscles, respectively, compared to the enzyme activity in food-deprived chickens.

Impact of nutritional states on S6K1 phosphorylation. There was weak phosphorylation of S6K1 in food-deprived chickens, whereas samples from chickens treated with insulin used as a positive control showed highly phosphorylated S6K1 (Fig. 3D, expt. 2). Both S6K1 serine and Thr 389 phosphorylations were induced from the first min of refeeding and were maintained for at least 30 and 60 min after the beginning of the 30-min refeeding period for serine and Thr 389 phosphorylation, respectively.

Activation of muscle S6K1 by insulin. The S6K1 band in gastrocnemius muscle was clearly shifted from 7 min after treatment, whereas gel retardation in pectoralis major muscle appeared only 15 min after insulin injection (Fig. 4A, expt. 3). S6K1 activity was measured 15 min after insulin treatment. Insulin increased S6K1 activity in both muscles compared to the enzyme activity in food-deprived chickens (Fig. 4B, **P < 0.001**). S6K1 activity was higher in gastrocnemius than in pectoralis major muscles in insulin-treated chickens but simi-
lar in both muscles in food-deprived chickens. The response to insulin therefore corresponded to an increase in S6K1 activity of about eight- and 11-fold in pectoralis major and gastrocnemius muscles, respectively.

**DISCUSSION**

In this study, S6K1 was characterized in chicken muscles. The partial cDNA sequence of chicken S6K1 showed high identity to its mammalian homolog. The chicken S6K1 had a similar molecular weight and cross-reacted with mammalian S6K1 antibody, which is not cross-reactive with p90<sub>rsK</sub>. Refeeding clearly induced phosphorylation and activation of S6K1, irrespective of the muscle studied (pectoralis major and gastrocnemius). In both muscle types, the magnitude of S6K1 activation after refeeding or insulin treatment was similar, even though the expression of S6K1 seemed higher in gastrocnemius than in pectoralis major muscle.

Furthermore, 30 min of refeeding was sufficient to induce S6K1 activation in both muscle types. This is consistent with the activation interval in cell culture (12). Short-term stimulation of muscle protein synthesis was previously reported after refeeding in chicks (22,23). Briefly, in starved chicks, the fractional rate of muscle protein synthesis was increased after 30 min refeeding, to reach values in the fed state. This was explained by changes in the amount of protein synthesized · d<sup>-1</sup> · unit RNA<sup>-1</sup> (i.e., changes in translational efficiency). Because mobility retardation of S6K1 is induced by food intake and is concomitant with stimulation of translation initiation in mammals (6,13), our results suggest that the S6K1 pathway could have a role in translation activation in chicken muscle.

It is noteworthy that S6K1 phosphorylation on serine residues and Thr 389 after refeeding occurred before the S6K1 enzyme activation. Changes in S6K1 activity in mammals are associated with the phosphorylation on serine and threonine residues (24,25). Moreover, phosphorylation of Thr 389 is most closely correlated with S6K1 activity in vivo (24). The correlation between S6K1 phosphorylation and S6K1 activity may indicate that similar mechanisms occur in chicken muscle. Finally, the present study showed that S6K1 activation was sustained during the first 2 h after feed withdrawal. This was consistent with a previous study in which S6K1 activity in rat muscle was maintained for 3 h after food withdrawal (13), and then progressively returned to the basal level within 9 h. Interestingly, 2 h after refeeding, both fractional synthesis rate and translational efficiency are maintained at the levels of well-fed control chickens (22).

We also showed that S6K1 activation occurred parallel with the increase in plasma insulin concentrations. The circulating insulin reached its maximum 30 min after refeeding and then progressively declined. S6K1 showed similar kinetics of activation, with a zenith at 30 min after refeeding. This is, to our knowledge, the first in vivo evidence showing a relationship between plasma insulin level and S6K1 activity. Furthermore, the injection of insulin to food-deprived chickens activated S6K1 despite the relative insulin resistance of chicken muscle to exogenous insulin (16) and the lack of a refeeding-related response of chicken muscle PI 3′-kinase (18). Thus, the insulin signaling pathway involved in the S6K1 activation needs to be clarified.

In conclusion, S6K1 is expressed in chicken skeletal muscle. This kinase is regulated by nutritional status and insulin in vivo despite the particularities of the insulin signaling pathway in chicken muscle.
**FIGURE 4** Effects of insulin on S6K1 gel mobility (A) and specific activity in pectoralis major and gastrocnemius muscles (B, exp. 3). S, food-deprived chickens; F, freely fed chickens. Results represent means ± SEM, n = 5. Values are expressed in Bq/500 μg total protein. Different from food-deprived chickens or different between muscle types for insulin-treated chickens, ***P < 0.001.

### LITERATURE CITED