Early post-hatching starvation delays p70 S6 kinase activation in the muscle of neonatal chicks

Karine Bigot¹, Mohammed Taouis¹,2, Michel Picard¹ and Sophie Tesseraud¹*  
¹Régulation du Métabolisme des Oiseaux, Station de Recherches Avicoles, Institut National de la Recherche Agronomique, Centre de Tours-Nouzilly, 37380 Nouzilly, France  
²Laboratoire de Biologie Cellulaire et Moléculaire, Biotechnologies, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas, France

(Received 17 February 2003 – Revised 23 June 2003 – Accepted 30 July 2003)

Chicken muscle ribosomal protein S6 kinase (S6K1) has been recently characterised and its enzymic activity is regulated by the nutritional and hormonal (insulin) status in vivo. The regulation of S6K1 is still unknown in neonatal chicks. The present study aimed to compare the activation of S6K1 in early-feeding (EF) and 48 h-delayed-feeding (DF) chicks from hatching to 4 d of age. During post-hatching starvation, S6K1 activity remained at the basal level measured in the control-hatched chicks. The maximum S6K1 activity was recorded on the first day of feeding with an increase of about 2·5-fold in the EF and DF chicks (P<0·01). S6K1 activity was correlated with plasma insulin level, suggesting a probable insulin-dependent S6K1 activation. The feeding-induced increase in S6K1 activity was related to its Thr389 residue phosphorylation. A similar pattern for protein kinase B phosphorylation was observed, upstream from S6K1. The S6K1 pathway was stimulated to the same extent in the EF and DF chicks, which indicates that post-hatching starvation did not increase S6K1 activation. It is concluded that muscle S6K1 is activated as soon as food is available without improvement in the response of the S6K1 pathway after post-hatching starvation.

Chicks: Neonatal feeding: p70 S6 kinase activation: Skeletal muscle

A key element in the control of protein synthesis is cytoplasmic serine–threonine kinase p70 S6 kinase (S6K1). S6K1 is responsible for the in vivo phosphorylation of ribosomal protein S6 (Ballou et al. 1991; Kawasome et al. 1998) and then it regulates the translation of specific mRNA (TOP mRNA) that encode essential components of the protein synthesis apparatus (Dufner & Thomas, 1999). Protein synthesis and S6K1 in mammals are positively regulated by the nutritional state implies that not only insulin, but also nutrients, including amino acids and particularly branched-chain amino acids, play a critical role both in vitro and in vivo (Long et al. 2000; Peyrollier et al. 2000; Kimball & Jefferson 2002; Proud 2002). However, the precise mechanism by which S6K1 is activated remains unclear in mammals as in other species.

The responsiveness to the nutritional status of skeletal-muscle protein synthesis and the insulin-signalling pathway is particularly pronounced during the neonatal period (Suryawan et al. 2001; Davis et al. 2002). In particular, feeding-induced S6K1 stimulation is significantly higher in 7-d-old than in 26-d-old piglets (Kimball et al. 2002). It has recently been shown that S6K1 is expressed in the breast muscle of 3-week-old chickens and is activated by both the nutritional state and insulin (Bigot et al. 2003b). S6K1 stimulation was observed despite the relative insulin resistance of chicken muscle to exogenous insulin (Simon 1989; Taouis et al. 1993) and the lack of a refeeding-related response of chicken muscle phosphatidylinositol 3'-kinase (Dupont et al. 1998). At the present time, the regulation of S6K1 remains undefined in chick neonates as well as the possible specificity in insulin action. The first week of life in chicks is characterised by intensive growth and tissue development with an increase of body weight by 2-fold (Chamblee et al. 1992; Murakami et al. 1992). Moreover, breast-muscle weight is also increased 5-fold during the first 4 d of age (Bigot et al. 2003a). During this critical period of 4 d, the residual yolk is completely resorbed and the enzymic functions of the gut are activated (Bigot et al. 2001). In practice, hatching and transportation procedures delay the feeding of chicks by

Abbreviations: DF, delayed-feeding; EF, early-feeding; IRS, insulin-receptor substrate; PKB, protein kinase B (also termed Akt); S6K1, p70 S6 kinase; Tris, (tri(hydroxymethyl)-amino)methane.

* Corresponding author: Dr S. Tesseraud, fax +33 2 47 42 77 78, email tesserau@tours.inra.fr
10 to 60 h. Nutrients in the residual yolk that are supposed to counter the lack of food during this starvation period represent an insufficient contribution to the nutritional requirements for both maintenance and growth in today’s fast-growing broiler chicks (Bigot et al. 2001). A delayed food supply reduces chick growth and final body weight (Noy & Sklan, 1999) and it has previously been reported that the early stages of muscle development are delayed as long as food is not available (Bigot et al. 2003a). The effect of a lack of access to food on the regulation of the initiation of muscle protein translation in neonatal chicks has not been examined. Therefore, the aim of the present experiment was to study S6K1 activation in neonate chicks and determine the possible effect of early post-hatching starvation on this activation. The food-deprived chicks remained 2 d after hatching without food before having access to food for the same period (2 d). To examine the possible S6K1–insulin interaction, changes in the glucose–insulin balance and in PKB, a kinase upstream from S6K1, were also investigated.

**Materials and methods**

**Chemicals**

Nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and the pre-made polyacrylamide solution protogel was from Prolabo (National Diagnostic, Gredignon, France). Bovine serum albumin (fraction V, radioimmunoassay grade) and protein A–agarose were purchased from Sigma Chemical Company (St Louis, MO, USA). Anti-S6K1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Thr389 S6K1, anti–protein kinase B (PKB) and anti-phospho-Ser473 PKB antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). [γ^32P]ATP was obtained from NEN Life Sciences (Zaventem, Belgium). The S6K1 assay kit was purchased from Upstate Cell Signaling (Lake Placid, NY, USA).

**Animals and experimental design**

The conditions of egg incubation, hatching and rearing have been described previously (Bigot et al. 2003a). Briefly, chicks were assigned to one of two nutritional conditions. The first group of chicks (n = 89) were fed ad libitum 6 h after hatching (the time required for chicks to dry) with a balanced starter diet containing 12.12 MJ/kg metabolisable energy and 220 g crude protein/kg (Table 1). This was the early-feeding (EF) group. The other group of chicks (n = 86) remained without food for 2 d, i.e. 54 h (48 + 6 h) after hatching before receiving the same diet. This was the delayed-feeding (DF) group. Water was freely available to all chicks after hatching. During the experimental period the average amounts of feed intake were 9.6, 16.1, 20.5 and 21.4 g/d per 100 g body weight in the EF chicks, and 0, 0, 18.2 and 18.5 g/d per 100 g body weight in the DF chicks at 1, 2, 3 and 4 d of age respectively.

<table>
<thead>
<tr>
<th><strong>Table 1. Composition of the experimental diet (g/kg)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong></td>
</tr>
<tr>
<td>Maize</td>
</tr>
<tr>
<td>Wheat</td>
</tr>
<tr>
<td>Soyabean protein 48</td>
</tr>
<tr>
<td>Soyabean oil</td>
</tr>
<tr>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
</tr>
<tr>
<td>Lysine-HCl</td>
</tr>
<tr>
<td>DL-Methionine</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Trace minerals and vitamins</td>
</tr>
<tr>
<td><strong>Calculated composition</strong></td>
</tr>
<tr>
<td>Metabolisable energy (MJ/kg)</td>
</tr>
<tr>
<td>Crude protein</td>
</tr>
<tr>
<td>Lysine (%)</td>
</tr>
</tbody>
</table>

At 6 h of age (age 0), ten chicks were used to determine plasma parameters and to study the breast muscle S6K1 pathway at the beginning of the experiment. These chicks were the control-hatched chicks. The same determinations were performed at 1, 2, 3 and 4 d of age (exact biological age, i.e. 24 ± 2, 48 ± 2, 72 ± 2, and 96 ± 2 h after the beginning of the experiment respectively) in the EF and DF chicks (n = 10 per treatment). After blood sampling, the chicks were killed by decapitation and the Pectoralis major muscles (left and right) were removed, quickly frozen in liquid N₂ and stored at −80°C. All experiments were carried out with due regard to the legislation governing the ethical treatment of animals, and investigators were certificated by the French government to carry out animal experiments.

**Determination of plasma glucose and insulin levels**

Plasma glucose levels were measured by the glucose oxidase method using an automated analyser (Beckman glucose analyzer 2; Beckman Instruments, Palo Alto, CA, USA). Plasma insulin levels were determined by radioimmunoassay with a guinea-pig anti-porcine insulin antibody using chicken insulin as the standard (Ruffier et al. 1998).

**Western blot analysis**

The frozen tissues were ground in liquid N₂. Powdered muscles (500 mg) were homogenised on ice with an Ultra-turrax homogeniser (LaboModerne, Paris, France) in buffer A. Buffer A contained NaCl (150 mmol/l), tri(hydroxymethyl)-aminomethane (Tris) (pH 7.4) (10 mmol/l), EDTA (1 mmol/l), ethylene glycol-bis (β-aminoethyl ether)-N, N, N, N'-tetra-acetic acid (1 mmol/l), triton X-100 (1 %, v/v), NP-40 (0.5 %, v/v), protease inhibitors and phosphatase inhibitors. The protease inhibitors were phenyl methyl sulfonyl fluoride (2 mmol/l), leupeptin (10 μg/ml) and aprotinin (10 μg/ml). The phosphatase inhibitors were sodium fluoride (100 mmol/l), sodium phosphate (10 mmol/l) and sodium orthovanadate (2 mmol/l). The homogenates were centrifuged at 1000 g for 30 min at 4°C and the supernatant fractions were then ultracentrifuged for 45 min at 150000 g. The supernatant...
fractions were sampled and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

For the Western blotting analysis, 40 μg muscle protein were separated on 10% (w/v) SDS-PAGE and then transferred to a nitrocellulose membrane. Membranes were blocked according to the manufacturer’s procedure for 1 h at room temperature. Each membrane was incubated with an antibody directed toward S6K1, phospho-Thr389 S6K1, PKB or phospho-Ser473 PKB. After washing, the membranes were incubated with a second antibody coupled to peroxidase (1/10 000) for 1 h at room temperature. The bands were visualised by an enhanced chemiluminescence Western blotting kit (Cell Signaling Technology, Beverly, MA, USA) and exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY, USA). The bands were quantified by Scion Image software (Scion Corporation, Frederick, MA, USA).

p70 S6 kinase assay

S6K1 activity was measured in the muscle extracts by immune kinase assay according to the procedure in the S6 kinase assay kit (Upstate Cell Signaling, Lake Placid, NY, USA). Briefly, the muscles were homogenised in S6 kinase assay kit (Upstate Cell Signaling Technology, Lake Placid, MA, USA) and exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY, USA). The bands were quantified by Scion Image software (Scion Corporation, Frederick, MA, USA).

Results

Glucose and insulin levels

In the EF chicks, glycaemia was increased from hatching to reach its maximum at 2 d of age (+48%; P<0.001), then declined at 3 d of age (P<0.05; Fig. 1 (A)). Glycaemia was similar in the DF and EF chicks from hatching to 2 d of age. There was also a decrease between 2 and 3 d of age, which was more marked in the DF chicks than in the EF chicks. Consequently, glycaemia was lower in the DF than in the EF chicks at 3 d of age.
Plasma glucose concentrations became similar in the DF and EF chicks at 4 d of age.

Insulinaemia in the EF chicks remained unchanged during the first 2 d of feeding in the EF group (Fig. 1 (B)). At 3 d of age it increased by about 0·8-fold \((P<0·05)\), and at 4 d of age insulinaemia returned to the levels measured on days 0, 1 and 2. In the DF chicks, post-hatching starvation induced higher glucose levels but lower insulin levels compared with the values measured in the control-hatched chicks \((P<0·01)\). Insulinaemia was thus approximately five times lower than in the EF chicks at the same age \((P<0·001)\). Since food was available, insulin levels increased by about 3·5-fold \((P<0·01)\), reaching levels similar to those in the control-hatched chicks, but remained lower than in the 3-d-old EF chicks \((P<0·01)\). Insulinaemia was similar in the DF and EF chicks at 4 d of age.

**Pectoralis major muscle p70 S6 kinase activity in neonatal chicks**

In the EF chicks, muscle S6K1 activity increased at 1 d of age (2·6-fold; \(P<0·001\)) compared with the enzymic activity determined in the control-hatched chicks (Fig. 2). On day 2, S6K1 activity was not different from that of the control-hatched chicks, even though a 1·8-fold stimulation was recorded \((P=0·19)\). S6K1 activity became higher on day 3 (2·3-fold; \(P<0·01\)) before declining at 4 d of age and returning to the values measured in the control-hatched chicks. S6K1 activity in the DF chicks was lower during the 2 d post-hatching starvation compared with the enzymic activity determined in the control-hatched chicks \((P<0·01)\). An increase only occurred at the beginning of feeding (i.e. 3 d of age) and this higher S6K1 activity \((P<0·01)\) was maintained to at least 4 d of age. The comparison of S6K1 activity after 1 or 2 d of feeding shows no significant difference between the EF and DF chicks (1-d-old EF chicks \(v.\) 3-d-old DF chicks, \(P=0·46\); 2-d-old EF chicks \(v.\) 4-d-old DF chicks, \(P=0·23\)).

**p70 S6 kinase phosphorylation on Thr389 in neonatal chicks**

S6K1 phosphorylation on the Thr389 residue in skeletal muscle of the EF and DF chicks was then investigated. The signal obtained with the phosphospecific antibody was normalised for total S6K1 content. The amount of S6K1 in neonatal chick muscle was increased from hatching to 4 d of age in the EF chicks \((P<0·01\) on days 3 and 4) but was unchanged in the DF chicks (Fig. 3). In the EF chicks, S6K1 phosphorylation on the Thr389 residue was increased by about 8-fold \((P<0·01)\) at 1 d of age compared with the Thr389 phosphorylation level determined in the control-hatched chicks. The phosphorylation status was clearly decreased on day 2.
Protein kinase B phosphorylation on Ser473 in neonatal chicks

In the present study, a significant correlation was observed when S6K1 activity was plotted against plasma insulin levels (\( y = 0.712 + 0.091x, R^2 = 0.27; P < 0.001 \)). To determine whether insulin-signalling events upstream from S6K1 were also enhanced in the chick neonates, PKB phosphorylation in skeletal muscle was examined. The anti-PKB/Akt antibody used in the present study to determine PKB content detects total levels of endogenous Akt1, Akt2 and Akt3 proteins and does not cross-react with other related kinases. This antibody is chicken reactive (Egea et al. 2001; Stephens et al. 2001). The anti-phospho-PKB antibody recognises PKB when it is phosphorylated on Ser473 and does not cross-react with PKB phosphorylated on other residues or with related kinases such as S6K1. The PKB phosphorylation intensity was normalised for total PKB content. As observed for S6K1 content, the amount of PKB in EF chick muscle was increased from hatching to 4 d of age (\( P < 0.05 \) on day 4) but was unchanged in the DF chicks (Fig. 4). The phosphorylation status of PKB in the EF group increased by 1.3-fold (\( P < 0.05 \)) at 1 d of age compared with the Ser473 phosphorylation level determined in the control-hatched chicks. Ser473 phosphorylation in 2-, 3- and 4-d-old EF chicks returned to the values measured in the control-hatched chicks (\( P \) from 0.12 to 0.35 according to age). In the DF chicks, PKB phosphorylation on Ser473 was maintained at the basal level during the 2 d post-hatching starvation. When food was available in the DF group (on days 3 and 4), PKB phosphorylation was higher than in the control-hatched chicks (about 1.3-fold increase; \( P < 0.05 \)). PKB phosphorylation was similar in the EF and DF chicks after 1 d of feeding (1-d-old EF chicks v. 3-d-old DF chicks; \( P = 0.17 \)) or after 2 d of feeding (2-d-old EF chicks v. 4-d-old DF chicks; \( P = 0.25 \)).

Discussion

In the present study, the possible changes in S6K1 activation were investigated in the breast muscle of neonatal chicks food-deprived for the first 48 h post-hatching (DF chicks) or with immediate access to food (EF chicks). S6K1 phosphorylation and activity were increased as soon as food was available, but exhibited very low levels during post-hatching starvation. A relatively similar pattern was observed for PKB, a kinase upstream from S6K1. Food-related S6K1 activation was comparable in the EF and DF chicks, suggesting that this pathway was not up regulated after the starvation period.

When food was available immediately after hatching (EF chicks), a significant increase in muscle S6K1 activity was recorded on days 1 and 3 compared with the control-hatched chicks. The evolution of S6K1 phosphorylation on Thr389 in the EF chicks was the same, in agreement with the assumption that phosphorylation of Thr389 is closely correlated to S6K1 activity in vivo (Weng et al. 1995; Dufner & Thomas 1999). Post-hatching starvation resulted in a lack of muscle S6K1 phosphorylation and activation. With regard to the well-established role of S6K1, the present study suggests that starvation during the critical post-hatching period limits the efficiency of mRNA translation in muscle. Nutrients in the residual yolk not used during fetal life may not be sufficient to induce S6K1 activation in muscle so as to maintain plasma insulin concentrations at the levels observed in the control-hatched chicks.
Furthermore, post-hatching starvation prevents the increase of ribosomal capacity (Cs, i.e. potential for protein synthesis) recorded in the muscle of chicks fed immediately after hatching (Bigot et al. 2003a), affects satellite cell proliferation (myogenic precursors present in the skeletal muscle; Haley et al. 2000), and increases myonuclear apoptosis (Mozdziak et al. 2002). All these results agree with the reduced muscle development in the DF chicks.

The finding that S6K1 was activated after the onset of feeding in both the DF and EF chicks indicates that the activation of S6K1 in neonate chick muscle is due to changes in nutritional and hormonal status rather than to an ontogenic effect per se. Interestingly, the feeding-induced increase in S6K1 activity and phosphorylation were sustained over a relatively long period of time (days rather than hours). This model of feeding in neonatal chicks is original since it is very different from the post-prandial stimulation after overnight food deprivation usually studied in the literature (Svanberg et al. 1997; Davis et al. 2000; Kimball et al. 2002). Because food is consumed throughout the day providing an almost continuous supply of nutrients in chickens (Muramatsu, 1990), there are no repeated cycles of post-absorptive and feeding states due to meals.

In the present study, a significant relationship was observed between S6K1 activity and plasma insulin levels (P<0.001). This is consistent with the fact that insulin regulates S6K1 activity in the skeletal muscles of 3-week-old chickens (Bigot et al. 2003b). In addition, the supply of food increased the phosphorylation of PKB which is an upstream signalling element of S6K1 related to insulin signalling in mammals (Burgering & Coffer, 1995). Taken together, these findings indicate that insulin is involved in S6K1 activation in chicken muscle, despite the fact that chicks exhibit particularities concerning insulin regulation. These particularities are: a relative resistance to exogenous insulin (Simon, 1989; Taouis et al. 1993), high plasma levels of glucose (2 g/l), and a lack of a refeeding-related response of the muscle phosphatidylinositol 3-kinase pathway (Dupont et al. 1998). These particularities are factors of progress to understand the dys-regulations observed in other species including man.

Although a correlation was found between S6K1 activity and insulinemia, S6K1 activation occurs at day 1 in the absence of any increase in plasma insulin. Other results also suggest that insulin regulation could be altered in neonatal chicks during the first days of age. In the present study, there was an increase in plasma glucose level until 2 d of age in the EF chicks without changes in insulinemia, suggesting a lack of maturity of pancreatic β-cells just after hatching. This finding is in agreement with the development of the glucose-dependent insulin secretion reported in mammals (Bliss & Sharp, 1994). On day 3, the reduced glycaemia observed in the EF chicks was related to an increase in plasma insulin concentrations. It is noteworthy that S6K1 activity and phosphorylation on Thr389 further increased at that age in the EF chicks, concomitantly with insulinemia. When food is available after 2 d of starvation (3-old DF chicks), a reduction of glycaemia also occurred parallel with an increase in insulinemia and was more pronounced than that observed in the EF chicks. This could indicate a greater insulin sensitivity of glucose metabolism induced by early malnutrition. An insulin-sensitising effect of energy restriction, undernutrition or poor fetal nutrition has previously been reported in mammals, with greater insulin-stimulated glucose uptake into skeletal muscle and adipocytes (Ozanne et al. 1996, 1997; Agote et al. 2001). Little is known about the cellular and molecular mechanisms underlying this process. An insulin-related increase in glucose transport caused by energy restriction occurred in insulin-receptor substrate (IRS)-1 knockout mice, implicating an IRS-1-independent mechanism (Gazdag et al. 1999). Furthermore, energy restriction did not change the insulin stimulation of IRS-1, IRS-2 or phosphotyrosine-associated phosphatidylinositol 3'-kinase activity in rat muscle (Dean et al. 1998; Davidson et al. 2002). Similarly, in the present study, the response of the S6K1 pathway to feeding was not enhanced after post-hatching starvation. Thus, the signal–transduction pathways need to be clarified to understand the alterations of metabolic regulations in chickens as in mammals.

In conclusion, S6K1 is regulated by food intake in the breast muscle of neonatal broiler chicks. The feeding-induced increase in S6K1 activity is associated with its phosphorylation on Thr389 and with PKB phosphorylation on Ser473. Moreover, the present results suggest that muscle S6K1 activation is at least partly insulin dependent. In neonate chicks, a delayed access to feed induces changes in the glucose–insulin balance, indicating possibly an increase in muscle insulin action. However, early post-hatching starvation defers muscle S6K1 activation without leading to any immediate compensatory stimulation. Elucidating these mechanisms has importance for fundamental physiology as well as for nutritional application.

Acknowledgements

The authors would like to thank Yves Jego (Hubbard-ISA, France) for providing the fertile pedigree eggs, Bernard Guillerm for animal care and Claude Bouchot and Michel Derouet for technical assistance (INRA, 37380 Nouzilly, France). K. B. was supported by a grant from Conseil Régional de la Région Centre, France.

References


