Early Posthatch Feeding Stimulates Satellite Cell Proliferation and Skeletal Muscle Growth in Turkey Poults

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ABSTRACT The effect of early posthatch feeding on skeletal muscle growth and satellite cell myogenesis was studied in turkey poults. Pouls were either fed immediately posthatch or food-deprived for the first 48 h and then refed for the rest of the experiment. Body and breast muscle weights were lower in the starved poults than in fed controls throughout the experiment ($P < 0.05$). Cultures of breast muscle satellite cells revealed significantly higher DNA synthesis in the fed group than in the starved group as early as d 1 ($P < 0.05$). These levels continued to rise, reaching ~500-fold those of feed-deprived poults on d 4. In the latter group, thymidine incorporation peaked only on d 6, and then declined. Thereafter, it decreased to the same levels as those in the fed group. Satellite cell number per gram muscle increased until d 4, and was higher in the fed group than in the starved group ($P < 0.05$). Pax7 levels in cell cultures derived from the fed group were markedly higher than in the starved group on d 2 ($P < 0.05$). Myogenin levels in both culture and muscle were higher in the fed than in the starved groups until d 4 ($P < 0.05$). Phosphorylation of the survival factor Akt and cyclin-dependent kinase inhibitor p21 levels were higher in cells derived from the fed group relative to those from the starved group 48 h posthatch ($P < 0.05$). Similarly, Akt phosphorylation and insulin-like growth factor I (IGF-I) levels were significantly higher in the muscles of the fed group ($P < 0.05$). Together, these results suggest that immediate posthatch feeding of poults is critical for satellite cell survival and myogenesis probably via IGF-I.

KEY WORDS: turkeys • starvation • growth • skeletal muscle • satellite cells

Skeletal muscle growth in posthatch birds is dictated by hypertrophy of and the accumulation of nuclei in muscle fibers. The latter process is attributed to myogenic precursor cells, located beneath the basal lamina of the fibers, called satellite cells (1). In response to external signals, these cells are capable of entering the cell cycle and proliferating, differentiating and either fusing into existing fibers or fusing with each other to form new fibers (2,3). Terminal differentiation of myoblasts during embryo development, as well as of satellite cells postnatally, involves the withdrawal of myoblasts from the cell cycle and upregulation of the muscle-specific helix-loop-helix MyoD family of proteins, which includes MyoD, myogenin, Myf5 and MRF4, and the myocyte enhancer-binding factor 2 [reviewed in (4,5)].

The proliferation of satellite cells is regulated by several growth factors and hormones, including fibroblast growth factor [reviewed in (6)], platelet-derived growth factor (7), growth hormone (8–10) and hepatocyte growth factor (11,12). These mitogens inhibit satellite cell differentiation, whereas insulin-like growth factor I (IGF-I) (13,14) induces this process and improves muscle hypertrophy via its downstream mediators, the phosphoinositide-3 kinase (PI3K) and Akt (13,14). Recently, we reported that IGF-I plays an important role in satellite cell proliferation and muscle growth in chicks that were treated with mild heat during their first days posthatch (15).

In chicks selected for meat production, the processes of proliferation and differentiation of satellite cells are transient, but crucial, occurring in the first days posthatch. In broilers, the period in which satellite cells proliferate and differentiate lasts only 1 wk posthatch; after this, the satellite cell population declines dramatically (15–17). This implies that factors affecting the accumulation of satellite cells during early posthatch determine mature muscle size later on. However, few studies have examined satellite cell dynamics in posthatch poults.

Under commercial conditions, turkey poult hatch during a window of ~36 h, and are removed from the hatchery only after almost all of the eggs have hatched. Hatchery procedures, distribution and transportation to farms involve more time, and it is therefore common for newly hatched poult to be without feed for 48 h or more. During this period the poult decrease in weight, due mainly to use of nutrients from the yolk, although other tissues are also utilized (18). Previous studies in birds have shown that posthatch starvation for the first 48 h of life depresses body growth (19) and small intestinal development (20). This period of starvation also decreases satellite cell proliferation and irreversibly retards skeletal muscle growth in broilers, supporting the key role of these cells in

1 Supported by the Israeli Poultry Marketing Board. O.H. is the Incumbent of the Vigeveni Senior Lectureship in Animal Sciences.
2 To whom correspondence should be addressed.
3 Abbreviations used: bFGF, basic fibroblast growth factor; BW, body weight; CDK, cyclin-dependent kinase; IGF-I, insulin-like growth factor I; PI3K, phosphoinositide-3 kinase.
dictating muscle size (17). A recent study showed that the smaller muscle size found in starved chicks is due to enhanced apoptosis of myonuclei (21). As in chicks, posthatch starvation has a severe effect on small intestinal development and growth rate in turkey poult (22). At marketing, body and breast weights were decreased (18). However, to date, the effect of early posthatch starvation on muscle growth in poult, in particular with respect to satellite cell proliferation and differentiation at a very early age, has not been studied. The different growth patterns of broilers and poult (23) may reflect the different myogenesis of satellite cells. Although earlier studies demonstrated that the mitotic activity of satellite cells in turkeys is age related (24,25), these studies focused on poult from 1 wk of age on. In this study we examined the effect of immediate posthatch feeding on satellite cell proliferation in the first days of age, and examined the changes in the levels of regulatory proteins and growth factors involved in myogenesis.

MATERIALS AND METHODS

Experimental procedures. Poults (British United Turkey, Bizar, Israel) were obtained from a commercial hatchery within 1 h of clearing the eggshell and transported within 30 min to the facility. Poults were divided into groups on the basis of body weight (BW), clearing the eggshell and transported within 30 min to the facility. Three groups were held in the transfer boxes for 48 h without access to feed and water. After 48 h, the latter group was removed from the boxes and allowed to consume feed and water ad libitum. Poults were maintained in floor pens in temperature-controlled rooms. The composition of the starter diet is presented in Table 1 (26). All experimental procedures were approved by the Animal Welfare Committee of the Faculty of Agriculture, The Hebrew University of Jerusalem.

Cell cultures. Skeletal muscle satellite cells were cultured from the pectoral muscle of poult at various ages as described by Halvey and Lerman (27) with slight modifications. In brief, tissues were removed from the chicks under sterile conditions, chopped and incubated with pronase (1.4 g/L) followed by trypsin (2.5 g/L). DNAse 1 (0.6 g/L) was added for an additional 10 min. Enrichment of satellite cell culture was achieved by several centrifugations and trituration of the resuspended pellet. Cells were then filtered through a mesh (200 μm) and preplated for 2 h on a plastic Petri dish. Cells were counted using a hemocytometer, and plated at 5 × 10^5 cells/cm^2 in Dulbecco’s modified Eagle’s medium supplemented with 100 μL/L horse serum on gelatin-coated (1 g/L) dishes, and maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2_. On all days, cells were prepared under exactly the same conditions from 6 g of breast muscle that had been pooled from the experimental birds. Immunostaining of the cells for desmin revealed the recovery of an enriched population of myogenic cells with <3% of those cells being nonmyogenic (data not shown).

Thymidine incorporation. DNA synthesis was assessed by [3H]thymidine incorporation (27). [3H]thymidine (New England Nuclear, Boston, MA) was added (74 kBq/well) for 2 h of incubation. Radioactivity in dissolved precipitates was counted using a Tri-Carb 1600CA scintillation counter (Packard, Downers Grove, IL). Equal plating efficiency was verified by determining cell numbers in parallel wells.

Immunoprecipitation. Muscle tissue protein extracts were incubated overnight at 4°C with a polyclonal antibody against basic fibroblast growth factor (bFGF; 1:60; R&D Systems, Minneapolis, MN) and then incubated for an additional 2 h with protein A Sepharose beads (Amersham Chemical, Bucks, UK). Samples were washed three times with PBS and subjected to Western blot analysis.

Western blot analysis. Western blot analysis was performed as described above. In brief, cells were scraped off the dishes in lysis buffer, and muscle tissue was homogenized with a Kinematica homogenizer (Lucerne, Switzerland) for 30 s on ice in the same lysis buffer. All extracts were sonicated and normalized for protein content (BCA kit, Pierce, Rockford, IL), and equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). Membranes were incubated overnight at 4°C with the appropriate primary antibodies, then washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin G (Zymed, San Francisco, CA). Proteins were visualized using enhanced chemiluminescence (Pierce). The following primary antibodies were used: anti-IGF-1 monoclonal antibody (1:3000; Upstate Biotechnology, Lake Placid, NY); polyclonal antibodies against chicken myogenin (1:6,000; a kind gift from Bruce Paterson, NIH, Bethesda, MD) and polyclonal anti-p21 (1:1,500; Pharmingen, San Diego); monoclonal antibody against phosphorylated Akt (1:1000; Promega, Madison, WI) and total Akt (1:1000; Cell Signaling, Beverly, MA); monoclonal antibody against Pax7 (1:100; Developmental Studies Hybridoma Bank, University of Iowa, IA); monoclonal antibody against tubulin (1:2500; Oncogene, Boston, MA) and polyclonal antibody against bFGF (1:1500; R&D Systems). Densitometric analysis was performed on bands using Gel-Pro software. Protein levels in each lane were normalized to the levels of protein loaded on that lane, or to the levels of α-tubulin as an internal standard. Protein levels in breast muscle were examined individually for each chick and the levels on each day are presented as percentages of feed-deprived poult.

Statistical analysis. Data were analyzed by two-way ANOVA using the General Linear Models procedures of SAS for effects of diet and age (29). Differences between means were examined using the Tukey test and differences were considered significant at P < 0.05 unless otherwise stated.

RESULTS

Body and breast muscle growth in poult with immediate and delayed access to feed. In chicks that were deprived of feed for 48 h posthatch, BW was reduced on d 2 (Fig. 1A, P < 0.05), and then rose again, becoming significantly higher than that at hatch from d 6 onward (P < 0.05). The BW of these chicks was significantly lower (P < 0.05) than that of fed poult until 19 d of age. Absolute (Fig. 1B) and relative breast muscle weights (Fig. 1C) were significantly higher in fed poult
until d 10 ($P < 0.05$). Thereafter, until d 19, the groups did not differ.

**Satellite cell proliferation is reduced upon food deprivation.** The immediate reduction in breast muscle weight, its growth retardation and its decreased percentage in feed-deprived poults suggested an immediate effect on satellite cell proliferation. Breast muscle from poults that were either immediately fed or feed-deprived for 48 h posthatch was sampled on various days after hatch and satellite cells were prepared. Cells were counted, and their ability to proliferate in vitro was evaluated after 1 d in culture by a thymidine incorporation assay. Already on d 1 posthatch, thymidine incorporation was less in the feed-deprived group compared with fed poults ($P < 0.05$; Fig. 2A; $P < 0.05$). Incorporation continued to decrease over the next 3 d, whereas that of the fed group increased and reached levels ~500-fold higher than in the feed-deprived poults on d 4. Thereafter, thymidine incorporation declined dramatically in the fed chicks. In the feed-deprived group, incorporation increased slightly on d 4 of age and peaked on d 6. Thereafter, it decreased to the same levels as in the fed group. Overall, thymidine incorporation declined after 6 d of age with activity reaching basal levels on d 16 (data not shown).

The number of satellite cells per gram muscle in fed poults was maximal at d 4 of age, then declined substantially from d 6 onward ($P < 0.05$; Fig. 2B). The number of satellite cells per gram muscle was lower in the feed-deprived poults and increased only after they had access to feed ($P < 0.05$). The number of cells per gram muscle in the feed-deprived group was maximal between d 4 and 6, declining by d 9 to the levels of the fed group.
Influence of immediate access to feed on myogenic regulatory factors. The effect of immediate feeding posthatch on Pax7 levels was measured in satellite cell cultures derived from the poults. Densitometry analysis of Pax7 normalized to the housekeeping protein $\alpha$-tubulin revealed substantially higher levels of Pax7 in the cells derived from the fed group than in those from the starved group (Fig. 3C, $P < 0.05$). Myogenin levels in the feed-deprived group peaked later, on d 6, and were greater than in the controls at this time ($P < 0.05$). Because few satellite cells were obtained after d 6, protein analyses were not conducted on subsequent days.

The relative level of myogenin in the muscle tissue reflected that of the cultured cells and was more than fourfold higher in the fed group than in the feed-deprived group on d 2 and 4 (Fig. 4; $P < 0.05$). On d 6 and 9, the relative levels of myogenin had decreased and did not differ between muscles of feed-deprived and fed poults.

Growth factor levels. The level of bFGF protein in the breast muscle of the fed poults tended to be higher ($P = 0.02$) than in feed-deprived poults on d 2 (Fig. 5A). On d 4, however, this pattern was reversed and the bFGF level in the feed-deprived group was more than four times higher than that of fed poults ($P < 0.05$).

The IGF-I level was three times higher in the muscle of fed than in feed-deprived poults on d 2 (Fig. 5B). This difference increased markedly on d 4. However, on d 6, IGF-I expression in the muscle was lower in the fed than in the feed-deprived poults ($P < 0.05$). On d 9, IGF-I expression tended to be higher in fed than in feed-deprived groups ($P < 0.1$).

Interestingly, Akt phosphorylation, which is mediated by PI3K, was more than two times greater in the muscle of fed poults compared with the feed-deprived group on d 2 (Fig. 6A; $P < 0.05$). In satellite cells prepared from 2-d-old fed chicks, phosphorylated Akt expression was more than four times higher than that in cells prepared from feed-deprived chicks.
In both muscle tissue and cell cultures, total Akt expression was not altered by food deprivation (data not shown).

**DISCUSSION**

Early posthatch feeding has been shown to improve body and muscle growth in both chicks and turkey poults (17,18). Moreover, early posthatch feeding had a rapid enhancing effect on the proportion of breast muscle weight, which was significant both close to hatch and through marketing (18). In this study, we focused on the effects of immediate access to feed during the early growth period of poults. In particular, we studied the mechanism(s) by which immediate access to feed alters skeletal muscle development during that period.

The significant increase in body as well as muscle growth due to the early feeding corroborates our previous studies on chicks and turkey poults (17,18). The higher percentage of breast muscle in the fed group in the first 10 d suggests a specific stimulatory effect of immediate access to feed on muscle growth. This stimulatory effect could be attributed to alterations in satellite cell proliferation and differentiation followed by changes in hypertrophy. Previous studies have revealed that satellite cell cultures are a reliable tool for evaluating muscle growth in posthatch chicks (15,17). This is supported by a recent study showing that cultured satellite cells isolated from steers can "remember" their in vivo environment (30). Therefore, in this study, we examined the kinetics of cultured satellite cell populations during the first days posthatch in fed and feed-deprived poults. Immediate access to feed enhanced satellite cell-cycle activity after 1 d, resulting in a significant difference between fed and feed-deprived poults. This was most pronounced on d 4 posthatch, after which satellite cell activity of the feed-deprived group began to increase. These extensive differences were reflected in the expression of Pax7 in these cells (Fig. 3A), corroborating earlier reports that Pax7 is expressed in proliferating satellite cells in adult muscle along with Myf5 and MyoD (31,32). Although the kinetics of satellite cell activity in feed-deprived and fed poults was generally similar to that in chicks (17), immediate posthatch feeding had a longer-lasting effect in poults, and recovery of satellite cell activity after 48 h posthatch starvation was slower. This was probably reflected at later stages of growth because despite the small differences

(Fig. 6B). In both muscle tissue and cell cultures, total Akt expression was not altered by food deprivation (data not shown).

**FIGURE 6** 
Akt phosphorylation in breast muscle (A) and satellite cells (B) of 2 d feed-deprived (S) and fed (F) poults. Upper panels: equal loading of proteins was verified by reprobing the blots with an antibody against α-tubulin. Bands identifying the relative levels of phosphorylated Akt were quantified by densitometric analysis and normalized to that of α-tubulin. For A and B, results are means ± SEM, n = 5 and 3, respectively. *Different from the starved group at that time. Lower panels: representative Western blot analysis for phosphorylated Akt on d 2.
observed in muscle weight in the feed-deprived and fed groups between d 10 and 20, a significant reduction in body and muscle weight in feed-deprived poults has been shown at marketing at 20 wk (18).

In general, the kinetics of satellite cell proliferation was somewhat slower in poults than in chicks. For instance, thymidine incorporation in chicks was nearly zero on d 8 of age, whereas in poults, it was still observed on d 9 in both fed and feed-deprived groups, indicating continued satellite cell proliferation. Previous studies have found mitotic activity of satellite cells until 8 wk of age in poults; however, at that age, it is less than one third that at 2 wk of age (25).

The kinetics of satellite cell numbers paralleled the cell activity in both feed-deprived and fed groups. Satellite cell numbers were higher in fed relative to feed-deprived poults during the first 4 d posthatch; however, the difference was less pronounced than that for thymidine incorporation. Satellite cell number calculated per gram muscle almost doubled between d 2 and 4 in the feed-deprived poults. This apparent increase could be due to myofiber atrophy, which is a general phenomenon in malnutrition.

Thymidine incorporation, as well as satellite cell counts, markedly declined on d 6 in the fed group, whereas in the feed-deprived group, these numbers remained relatively high. This suggests that a substantial part of the satellite cell population in the former group underwent differentiation earlier than in the latter. Indeed, higher levels of myogenin were present in the muscle and in cultured satellite cells derived from the fed group on d 2 and 4, reflecting the differentiation process. In contrast, in feed-deprived poults, myogenin levels increased from d 6. Myogenin expression has been attributed to satellite cells in skeletal muscle and is upregulated during terminal differentiation of satellite cells (8,15,33,34). The early induction of myogenin levels in the fed group in culture and in vivo, could be because myogenin is an early regulatory factor that is upregulated in cycling cells, which then undergo terminal differentiation (35).

Previously, it was reported that the induction of the CDK inhibitor p21 coincides with or follows that of myogenin during differentiation (35,36). Surprisingly, the levels of p21 were highest on d 2 in satellite cell cultures derived from both feed-deprived and fed groups, whereas myogenin levels were at their lowest (Fig. 3B and C). It should be noted that in contrast to myogenin, p21 levels in the muscle were not consistent with those in satellite cell cultures (data not shown), likely because p21 is abundantly expressed, whereas myogenin expression is specific to differentiating satellite cells (33).

On d 2, p21 levels were substantially higher in the fed poults relative to those in the feed-deprived group. The p21 protein has been shown to be a specific substrate of the antiapoptotic agent Akt (37), and in vitro studies have suggested that both proteins play a crucial role in muscle cell survival (38,39). Moreover, in those studies it was reported that Akt-mediated cell survival is upregulated by IGF-I. Indeed, in the present study, Akt phosphorylation levels were in both satellite cell cultures and muscle, as well as IGF-I levels, were significantly higher in the fed relative to the feed-deprived poults on d 2 (Figs. 5B and 6), suggesting better survival of satellite cells in the fed group, at least in the early days posthatch. This suggestion is supported by a recent report showing that apoptosis occurs in skeletal muscles of chicks that were feed-deprived for 2 d posthatch (21). Under these conditions, total Akt levels were not altered, suggesting that Akt regulation occurs at the post-translational level. Together, these results suggest that at least in poults, and maybe in other birds, early posthatch feeding increases the survival of satellite cells via IGF-I-mediated induction of Akt phosphorylation. The up regulation of Akt phosphorylation may indeed lead to the induction of p21 (37), which was shown to be involved in muscle cell survival (38,39).

The levels of bFGF were higher in the skeletal muscles of the fed poults compared with feed-deprived poults on d 2, suggesting an induction of satellite cell proliferation in the fed poults. Both bFGF and IGF-I have been shown to stimulate satellite cell proliferation in rats (40,41) and broiler chicks (9,42,43), as well as in turkeys (44,45). However, in contrast to bFGF, IGF-I levels in muscles derived from fed poults approximately tripled those in the feed-deprived poults on d 2, and continued to rise on d 4. These findings suggest that IGF-I plays a major role in the survival and proliferation of satellite cells in the fed group. Moreover, it affects the earlier differentiation of satellite cells in the fed poults, probably via its direct effect on the PI3K pathway (13,14) and myogenin induction (6). We propose that locally expressed IGF-I causes the higher proliferation and differentiation of satellite cells in the fed poults. Recently, we reported that accelerated satellite cell myogenesis upon mild heat exposure of chicks at an early age is mediated specifically by local production of IGF-I, correlating with higher levels of myogenin (15). Other studies have also reported that IGF-I is the major local growth factor, playing a role in both muscle growth and hypertrophy via its effect on satellite cells (34,46,47). Notably, although those studies showed the effect of IGF-I directly or under various stress conditions, the present study is the first to show its effect due to nutritional status.

We conclude that the provision of exogenous feed and water to poults immediately posthatch is critical for optimal muscle development and growth. We suggest that the immediate feed intake leads to an increase in the levels of local growth factor in the skeletal muscle, which in turn affect satellite cell myogenesis and survival. Our results support the concept that IGF-I is a major growth factor in this process.

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LITERATURE CITED


