The Effects of Protein Restriction on Insulin-Like Growth Factor-I and IGF-Binding Proteins in Chickens (44299)

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Abstract. **The effect of dietary protein restriction (5% and 10% compared to control 20%) on circulating concentrations of insulin-like growth factor (IGF)-1 and IGF binding proteins (IGFBPs) was examined in young (18-day-old) chickens during a 2-week period. Reductions in dietary protein caused progressive growth retardation as evidenced by decreased body-weight gain and reduced bone growth. The decrease in plasma concentrations of IGF-I appeared to be directly related to dietary protein levels (i.e., the lower the amount of dietary protein, the greater the reduction in the circulating concentrations of this growth factor). Three IGFBPs with MWs of 30, 36, and 40 kDa were detected by radioligand assay following separation by SDS-electrophoresis. Binding activity of the 30-kDa IGFBP was transiently increased on Day 3 by protein restriction (both 5% and 10%). The 36-kDa IGFBP was also affected by protein restriction with binding activity of this IGFBP decreased throughout the study. The binding activity of the 40-kDa IGFBP was transiently increased on Days 3 and 7 but subsequently decreased on Days 10 and 14 in the 10% protein group. In the 5% protein group, binding activity of the 40-kDa IGFBP was decreased throughout the study. The decrease in circulating concentrations of IGF-I appeared to be inversely related to the initial increase in the binding activity of the 30-kDa IGFBP on Day 3 (5% and 10% protein) and to the increased binding activity of the 40-kDa IGFBP on Days 3 and 7 (10% protein). This may suggest that bioavailability of plasma IGF-I is decreased initially due to increased binding with these IGFBPs. However, binding activity of all three IGFBPs then decreased in a manner directly related to the decreasing IGF-I plasma concentrations for the remainder of the experiment. The initial increase in binding activity observed with the 30-kDa IGFBP is similar to that observed with IGFBP-1 in mammals in that both of these IGFBPs respond rapidly to nutritional deprivation. Decreased protein intake would certainly have an iimpact on the amount of available proteins required for the synthesis of these growth factors.**

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Circulating concentrations of insulin-like growth factor-I (IGF-I) have been shown to be influenced by nutritional factors such as dietary protein. Dietary protein restriction decreases IGF-I plasma concentrations in humans (1), rats [cerebral binding; during gestation and postnatally (2–4)], and chickens (5–7). Starvation or feed restriction also results in a decrease in plasma concentrations of IGF-I in a variety of species including humans (8–10), pigs (11), rats (12–16), and chickens (17). Although a decrease in dietary energy intake causes a reduction in plasma concentrations of IGF-I, this decrease can be prevented in humans through the administration of a diet high in protein (18). Conversely, administration of high-fat and

high-carbohydrate diets also results in a reduction in plasma concentrations of IGF-I in humans (18). In many species including humans, sheep, cattle, dogs, rats, and chickens, nutritional energy restriction results in reductions in circulating concentrations of IGF-I and concomitant increases in circulating concentrations of GH due to the loss of negative feedback from IGF-I (19–23). The present study examines the effects of varied decreasing amounts of protein in the diet on plasma concentrations of IGF-I, and the IGF binding protein (IGFBPs), in young chickens.

Six IGFBPs have been identified definitively. Three IGFBPs have been identified in the chicken, based on the ability of the IGFBPs to bind ^{125}I-human IGF-I (24–28) with an additional IGFBP identified using ^{125}I = clGF-II as a radioligand (29, 30). Although the specific function of each IGFBP has not been fully established, several roles have been suggested. They may serve as a transport mechanism for IGF-I in the blood and may limit the access of IGF-I to its cellular receptors. Other suggestions include a mechanism for limiting the inclusion of IGF-I into intracellular compartments and modulation of IGF-I tissue specificity (31, 32). Fasting can affect the plasma levels of IGFBPs in mammals [rats (12–14, 33), sheep (34), guinea pigs (35, 36), and chickens (28, 37). The 30-kDa IGFBP in chickens has been identified tentatively as IGFBP-2 since it showed weak antibody cross-reactivity with bovine IGFBP-2 in Western blot analysis (28). This 30-kDa IGFBP also behaves similarly to the human IGFBP-2 following hypophysectomy (28). However, the 30-kDa IGFBP may also share characteristics with mammalian IGFBP-1. Mammalian IGFBP-1 responds rapidly to changes in metabolic status caused by nutritional deprivation or fasting (38–40). Circulating levels of IGFBP-1 and -2 in mammals have been shown to increase during protein deprivation (14).

The present study examines the effects of protein restriction on plasma concentrations of IGF-I and the binding activity of IGFBPs in young growing chickens. The purpose of this experiment is to define the relationship between dietary protein availability and components of the IGF-I system.

Materials and Methods

Animals. One-day-old male white Leghorn male chicks were obtained from Avian Services, Inc. (Frenchtown, NJ). These were subsequently maintained on a commercial feed (Country Feeds Broilermaker, crude protein 21% min, crude fat 3% min, and crude fiber 4% max, Agway, Syracuse, NY) until 18 days old. At this time, chicks were assigned to three treatment groups containing nine chickens per group. Each of the groups was then assigned to one of the three treatments by random assignment. Animals in poor health or with body weights outside an acceptable range (mean ± 2 SDs) were not assigned to the study.

The three groups of 18-day-old chicks received specifically formulated chick diets containing 5%, 10%, or 20% soy protein (Diet Numbers D51031-5%, D51032-10%, and D51012A-20%, Research Diets, Inc., New Brunswick, NJ). Since the majority of NRC approved commercial feed preparations contain approximately 20% protein, the D51012A diet (20% protein) served as a control. All three diets used met NRC requirements with the exception of protein content. Body weights and shank-toe bone length (as an indicator of skeletal growth) were determined on Days 0 (pretest), 3, 7, 10, and 14. Food consumption was determined on a daily basis. Blood samples were obtained by venipuncture from the brachial vein on Days 0, 3, 7, 10, and following decapitation on Day 14. Average daily feed intakes per chicken in the control group (20% protein) and in the groups receiving 10%, and 5% protein were 54.2, 58.9, and 26.3 g, respectively, per day. All the chickens were sacrificed following 14 days of treatment.

IGF-I Radioimmunoassay. Plasma concentrations of IGF-I were determined by a heterologous radioimmunoasssay [(41), as validated for chicken plasma: 42]. Plasma concentrations of IGF-I were determined in duplicate in single assays in order to eliminate interassay variance. The coefficient of variation for the IGF-I assay was 13.3%.

IGFBP Analysis. Plasma IGFBPs were identified by and subsequently quantified using radioligand blotting techniques that were slightly modified from those of Hossenlopp *et al.* (43, 44). The preparation of all plasma samples included an acid extraction prior to analysis in order to dissociate the IGFBPs from the ligand. In addition, the evaluation of non-specific binding of the IGFBPs had been previously performed in our laboratory [i.e., the addition of nonradioactive IGF-I in excess completely inhibited the binding of the radioactive ligand (36)]. Molecular sizes of the isolated IGFBPs were estimated using prestained SDS-PAGE low-range standards (Bio-Rad Laboratories, Hercules, CA). Briefly, this entailed the following. Plasma samples (10 μl of plasma combined with 25 μl of sodium dodecyl sulfate (SDS) sample buffer (see below) were boiled for 5 min. Proteins were separated by SDS discontinuous slab polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-PROTEAN II Ready Gels, Bio-Rad Laboratories, Hercules, CA; 4% stacking and 12% separating acrylamide gels) under nonreducing conditions. The sample buffer contained 10% glycerol, 12.5% 0.5 *M* Tris-HCl pH 6.8, 2% SDS, and 0.00125% bromophenol blue. Following electrophoresis, the separated proteins were electrotransferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) for 1 hr at 100 V using a transfer buffer containing 20% methanol, 25 m*M* Tris, and 192 m*M* glycine pH 8.3. Following electrotransfer, the membranes were dried at 37°C, then washed for 30 min in a ligand blot buffer containing 0.15 *M* NaCl, 0.01 *M* Tris pH 7.4, 0.5 mg/ml sodium azide, and 3% Nonidet P-40 (Sigma, St. Louis, MO). Membranes were then blocked with 1% bovine serum albumin (BSA) (Intergen, Purchase, NY) in Tris buffered saline (TBS) for 2 hr followed by a 10-min wash with TBS plus 0.1% Tween 20 (TTBS, Bio-Rad Laboratories). The nitrocellulose transfer membranes were incu-

Table I. The Effects of Protein Restriction on Body Weight in White Leghorn Chickens

Treatment (percentage of protein in formulated diet)	Body weight (g)† Duration of treatment (days)				
	0	3	7	10	14
20	183.8 ± 3.1[a]	212.7 ± 5.0[a]	269.4 ± 6.7[a]	316.3 ± 7.3[a]	375.9 ± 8.5[a]
10	184.7 ± 2.9[a]	197.2 ± 4.6[b]	242.2 ± 5.6[b]	274.8 ± 7.3[b]	313.7 ± 9.2[b]
5	184.6 ± 3.0[a]	181.0 ± 3.2[c]	182.4 ± 3.4[c]	180.6 ± 2.7[c]	183.0 ± 3.9[c]

† Values represent means ± SEM, $n = 9$ chickens/group for all groups.
[a–c] Means with different superscript letters differ ($P < 0.05$) by Analysis of Variance (ANOVA), followed by Least Significant Differences (LSD).

bated overnight with ^{125}I-human IGF-I (Amersham, Arlington Heights, IL; 300,000 cpm/ml) in TTBS contining 1% BSA. Subsequently, membranes were washed multiple times with TBS and TTBS to remove unbound ligand. All incubations were performed at 4°C. Blots were then dried at 37°C and placed in a film cassette containing intensifying screens and x-ray film (Hyperfilm MP, Amersham). The films were exposed for 3–7 days at −80°C. Radiographs were analyzed using laser densitometry (Pharmacia, Piscataway, NJ) to quantify the approximate relative changes in IGFBPs. All radiographs contained a control lane consisting of a pooled sample of 1- and 27-day-old chicken plasma (this same standard plasma pool was used for all radioligand assays). The IGFBPs from plasma samples were compared to those found in the standard plasma pool. All mentions of "standard" in the following text concerning binding proteins refer to the pooled plasma "standard." Data are presented as a percentage of the optical density (OD) observed for the standard plasma pool.

Statistical Analysis. Data were analyzed by analysis of variance (ANOVA). Significant differences in means were determined by least significant differences (LSD). Statistical analysis was performed using Excel, Version 5, Analysis ToolPak.

Results

Growth (Body Weight, Shank-Toe Bone Length). As expected, chickens in the control group (receiving the 20% protein diet) exhibited steady increases in body weight throughout the experiment. The groups receiving the protein-deficient diets showed reduced ($P < 0.05$) body weight compared to the controls given the 20% protein diet (Table I). However, the chickens receiving 5% protein showed no body-weight gain during the 14-day observation period. Reductions in bone growth ($P < 0.05$) were noted in both the 5% and 10% protein groups compared to the 20% protein group by Day 3 (Table II), and these became greater in magnitude as the study continued. Although there was no change in body weight observed in the 5% protein group, bone lengths did increase during the study, as they did in the 10% protein group, albeit not as substantially as in the control 20% protein group. Chickens in the 10% and 5% protein groups showed a 2% and 4% decrease ($P < 0.05$) in shank-toe length by Day 3, progressing to an 8% and 18% decrease on Day 14, respectively.

Circulating Plasma Concentrations of IGF-I. Plasma concentrations of IGF-I in chicks of the control 20% protein group increased steadily between Day 7 and Day 14 of the experiment. In chicks on 10% protein, plasma concentrations of IGF-I were decreased ($P < 0.05$) compared to the 20% protein control but did not differ from pretreatment on Days 3, 10, and 14 (Fig. 1). An even greater reduction ($P < 0.05$) in plasma concentrations of IGF-I was observed throughout the experiment in the 5% protein group. Plasma concentrations of IGF-I were 20%–35% lower in the 10% protein group and 46%–68% lower in the 5% protein group between Days 3 and 14 compared to the 20% protein controls.

Binding Activity of IGFBPs. In this experiment, three IGFBPs binding proteins were characterized using SDS-PAGE electrophoresis. Radiolabeled ^{125}I-human IGF-I bound to specific proteins with MWs of approximately 30, 36, and 40 kDa (Fig. 2).

Table III summarizes the binding activity of the 30-kDa

Table II. The Effects of Protein Restriction on Shank-Toe Bone Length in White Leghorn Chickens

Treatment (percentage of protein in formulated diet)	Shank-toe bone length (cm)† Duration of treatment (days)				
	0	3	7	10	14
20	8.0 ± 0.5[a]	8.6 ± .06[a]	9.5 ± .07[a]	10.1 ± .08[a]	10.7 ± .08[a]
10	8.1 ± .05[a]	8.4 ± .08[b]	8.9 ± .12[b]	9.4 ± .09[b]	9.8 ± .10[b]
5	8.0 ± .04[a]	8.3 ± .05[b]	8.6 ± .05[c]	8.7 ± .05[c]	8.8 ± .08[c]

† Values represent means ± SEM, $n = 9$ chickens/group for all groups.
[a–c] Means with different superscript letters differ ($P < 0.05$) by Analysis of Variance (ANOVA), followed by Least Significant Differences (LSD).

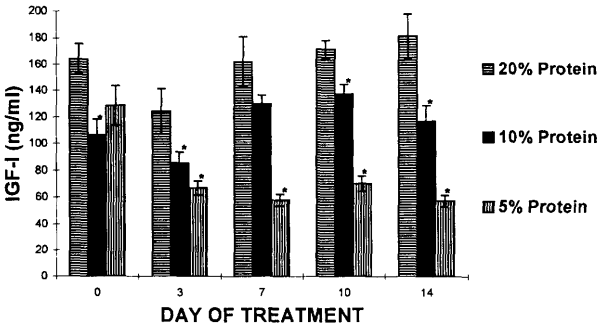

Figure 1. The effects of dietary protein restriction on plasma concentrations of IGF-I in chickens receiving 5, 10, and 20% protein in the diet. The values are means ($n = 6$) ± SEM. *$P < 0.05$.

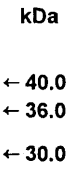

Figure 2. Representative ligand blot of chicken plasma IGF-binding proteins (IGFBP). Plasma proteins were separated by electrophoresis and blotted with ^{125}I-labeled IGF-I as outlined in the text. Proteins with approximate molecular masses of 30, 36, and 40 kDa were identified. Each blot contained a plasma standard in the Lane 1 position (far left lane).

IGFBP in chicks receiving the 5%, 10%, and 20% protein diets. On Day 3, the binding activity of the 30-kDa IGFBP was dramatically increased ($P < 0.05$) in the 5% and 10% protein groups (respectively to 360% and 250% of controls). This was not observed on Days 7 and 10.

The most prevalent IGFBP was that with a MW of approximately 36 kDa. The binding activity of the 36-kDa IGFBP was decreased ($P < 0.05$) (Day 7) or tended to be reduced in the 5% protein group beginning on Day 3 and continuing throughout the end of the experiment (Table IV). Moreover, the binding activity of the 36-kDa IGFBP was decreased on Day 7 in the 10% protein group. The second most prominent IGFBP was that with a MW of approximately 40 kDa (Fig. 2). Binding activity of the 40-kDa IGFBP was increased ($P < 0.05$ on Day 7) in chicks on both the 5% and 10% diet.

Discussion

Young cockerels experiencing rapid growth were chosen for this experiment since effects on growth and its endocrine control might be expected to be more evident. As might be expected, reductions in dietary protein resulted in decreases in body and skeletal growth (Tables I and II). Plasma concentrations of IGF-I were reduced in the 5% and to a less extent in the 10% protein groups (Fig. 1). Reduced plasma concentrations of IGF-I have been reported previously in protein-restricted chickens (5) and rats (4). Similarly, reductions in circulating plasma concentrations of IGF-I occur during fasting (28) or even mild feed restriction (40%, 60%, and 80% of control *ad libitum* diet) in young chicks (37). Reduced circulating concentrations of IGF-I induced by nutritional insufficiency in chickens are associated with increased GH synthesis (17) and release (19) presumably reflecting removal of IGF-I negative feedback. Since increased GH secretion during feed restriction appears to have little or no effect on preventing either skeletal or body-growth retardation in young chickens (the GH-IGF-I axis being uncoupled), growth is much more dependent upon normal IGF-I levels (42, 45). In the present study, skeletal growth retardation, marked reductions observed with 5% protein but only modest decreases in the 10% protein group, appeared to be linked to plasma concentrations of IGF-I.

In the present and previous studies with chicken plasma, three IGFBPs were observed (Fig. 2, Refs. 24, 27, 28, 37). The binding activity of the 30-kDa IGFBP appeared to be sensitive to various physiological changes such as hyperthyroidism, hypophysectomy (46), feed restriction (energy/protein) [37, protein transitorily-Table III], and fasting (28). The 30-kDa IGFBP in chickens has also been tentatively identified as IGFBP-2 based on its cross-reactivity with antisera to bovine IGFBP-2 in Western blot analysis (28). However, the physiological changes in the 30-kDa IGFBP in the chicken show similarities to those observed in mammals for both IGFBP-1 and -2. The binding activity of IGFBP-1 in mammals increases rapidly following nutritional deprivation (energy/protein restriction) or fasting (38–40). Protein restriction causes an increase in both serum IGFBP-1 and -2 in mammals (14). Increases in

Table III. The Effects of Dietary Protein Restriction on the 30-kDa IGF Binding Protein in White Leghorn Chickens

Treatment (percentage of protein in formulated diet)	Optical density† (% of standard plasma) Duration of treatment (days)				
	0	3	7	10	14
20	250.4 ± 85.0[a]	81.3 ± 6.4[a]	195.8 ± 74.1[a]	234.1 ± 69.9[a]	105.8 ± 47.0[a]
10	149.3 ± 41.3[a]	203.0 ± 34.3[b]	160.3 ± 77.9[a]	101.5 ± 31.1[a]	108.8 ± 24.7[a]
5	179.4 ± 48.9[a]	292.4 ± 37.7[b]	93.0 ± 32.1[a]	91.1 ± 28.6[a]	97.8 ± 22.5[a]

† Values represent means ± SEM, $n = 6$ chickens/group for all groups.
[a–b] Means with different superscript letters differ ($P < 0.05$) by Analysis of Variance (ANOVA), followed by Least Significant Differences (LSD).

Table IV. The Effects of Dietary Protein Restriction on the 36-kDa IGF Binding Protein in White Leghorn Chickens

Treatment (percentage of protein in formulated diet)	Optical density† (% of standard plasma) duration of treatment (days)				
	0	3	7	10	14
20	65.2 ± 5.4[a]	62.2 ± 6.8[a]	86.6 ± 4.6[a]	71.4 ± 13.3[a]	112.2 ± 9.6[a]
10	60.8 ± 4.4[a]	48.2 ± 6.6[a]	64.9 ± 5.5[b]	70.9 ± 15.8[a]	104.9 ± 13.0[a]
5	79.1 ± 7.6[a]	54.1 ± 4.4[a]	65.0 ± 3.5[b]	52.6 ± 4.2[a]	86.3 ± 6.7[a]

† Values represent means ± SEM, $n = 6$ chickens/group for all groups.
[a–b] Means with different superscript letters differ ($P < 0.05$) by Analysis of Variance (ANOVA), followed by Least Significant Differences (LSD).

Table V. The Effects of Dietary Protein Restriction on the 40-kDa IGF Binding Protein in White Leghorn Chickens

Treatment percentage of protein in formulated diet)	Optical density† (% of standard plasma) Duration of treatment (days)				
	0	3	7	10	14
20	111.3 ± 8.6[a]	219.2 ± 7.4[a]	256.0 ± 46.5[a]	143.2 ± 13.0[a]	336.4 ± 41.0[a]
10	149.5 ± 9.9[a]	256.1 ± 29.7[a]	271.9 ± 61.8[b]	112.5 ± 10.5[a]	271.7 ± 29.5[a]
5	176.7 ± 26.1[a]	239.5 ± 20.6[a]	217.2 ± 56.5[b]	92.5 ± 10.2[a]	194.1 ± 39.6[a]

† Values represent means ± SEM, $n = 6$ chickens/group for all groups.
[a–b] Means with different superscript letters differ ($P < 0.05$) by Analysis of Variance (ANOVA), followed by Least Significant Differences (LSD).

IGFBP-1 and -2 mRNA have also been observed during malnutrition (protein restriction) in neonatal rats (47).

Binding activity of the 36-kDa IGFBP was also influenced by protein restriction (Table IV). Unlike the 30-kDa IGFBP, no initial increase in the binding activity of the 36-kDa IGFBP was observed. In contrast, the binding activity was decreased (Day 7) or tended to be decreased in both the 5% and 10% protein groups during the majority of the experiment. This result is similar to the response to feed (energy/protein) restriction in chickens where, following a transitory increase in the 36-kDa IGFBP, a decrease was observed subsequently (37). However, the difference is that the decrease in the present study was observed at a much earlier stage. This suggests that restriction of specific dietary components (i.e., protein) may affect the binding activity of the 36-kDa IGFBP. The increases in the binding activity of the 36-kDa IGFBP with feed (energy/protein) restriction (37) is similar to that observed with IGFBP-1 and -2 in mammals (14, 38, 40). However, this was not the case with protein restriction (Table IV). It may be speculated that the reductions in the plasma concentrations of IGF-I and the 36-kDa IGFBP reflect a similar uncoupling of GH control, perhaps by loss of GH receptors.

The 40-kDa IGFBP was little affected by dietary protein (Table V). Mammalian IGFBP-3 has a similar MW (40 kDa) as the 40-kDa IGFBP found in chickens (48). Mammalian IGFBP-3 is largely unaffected by metabolic status in rats (47) and the humans (49). However, conflicting findings have shown decreases in serum IGFBP-3 and liver IGFBP-3 mRNA in young and neonatal rats (4, 14, 47, 50). Also in contrast, a decrease has been noted in kDa-3 in porcine serum following fasting (11).

In conclusion, dietary-protein restriction influences both plasma concentrations of IGF-I and IGFBPs. The changes in IGFBPs will influence the availability of circulating IGF-I in the plasma and hence to tissues.

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