Insulin-like growth factor binding proteins in rats respond to fasting and the protein and energy content of the re-feeding diet

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We investigated the effects of fasting and re-feeding various levels of protein and energy on serum levels of specific insulin-like growth factor binding proteins (IGFBPs) and serum IGF-I binding activity patterns in rats. Fasting (48 hr) caused serum IGF-I levels to decrease 62% from 400 ng/mL to 151 ng/mL ($P < 0.05$). Re-feeding fasted animals (48 hr) a low protein-low energy diet (control diet, intake 66% lower than re-fed control group) maintained IGF-I at fasted levels whereas re-feeding a low protein-isocaloric (5% protein, energy-adequate) diet caused IGF-I levels to drop 50% below fasted levels to 76 ng/mL ($P < 0.05$). IGF-I binding activity in the 155K region paralleled serum IGF-I levels. Fasting caused a 150% increase in IGF-I binding activity in the 40K region. Re-feeding a control diet restored both peaks of IGF binding activity to baseline levels. IGF binding activity in the 40K region of rats re-fed a low protein-isocaloric diet increased 60% above fasting (over 200% above control). Energy level with protein restriction was important since responses in both IGF binding peaks were greater in low proten-isocaloric than low protein-low energy re-fed groups. Ligand blotting revealed that protein restriction at both energy levels caused the 38–45K bands (IGFBP-3) to decrease, and the 32K band (IGFBP-1) to increase compared to baseline, fasted, or re-fed control levels. Signal intensity of the IGFBP 24K band was lower in fasted and low protein-low energy rats compared to groups receiving adequate energy. Thus, that IGFBPs are differentially regulated and acutely responsive to nutritional manipulations suggests that the IGFBPs may be markers of acute changes in nutritional status.

Keywords: insulin-like growth factor-I; IGF binding proteins; protein-energy deficiency

Introduction

The insulin-like growth factors (IGFs) are potent growth regulators that are under endocrine and nutritional control.[1-5] The IGFs are known to circulate in extracellular fluids bound to specific carrier proteins. These binding proteins are an integral component of IGF-mediated growth regulation and have been reported to alter cellular responses to the IGFs. Both inhibition and enhancement of IGF actions have been demonstrated by IGF binding proteins.[6-10]

Three major IGF binding proteins (IGFBPs) have been characterized and designated, IGFBP-1, IGFBP-2, and IGFBP-3.[11] In the blood, the IGFBPs can be separated into 150K and 40K size classes. In rats, the 150K IGFBP region contains IGFBP-3, which is growth hormone (GH) dependent.[12,13] The 40–50K region contains a protein of 32K by ligand blotting that is the rat homolog of IGFBP-1.[14] IGFBP-1 is independent of GH secretion and increases with fasting in humans.[15-17] IGFBP-2, which is approximately 30K in

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size, is primarily a fetal binding protein.[12,13] The nature of a 24K protein from the 40–50K region remains unclear.[13] The influence of nutrition on the IGFBPs in rats bears further investigation.

Protein deficiency attenuates circulating IGF levels and bioactivity.[2,3,5,18-21] The metabolic adaptation to protein deficiency is profoundly influenced by the availability of energy as is evident in the distinct clinical and biochemical conditions of kwashiorkor versus marasmus.[22]

In this paper we report that fasting and re-feeding a diet inadequate in protein and/or energy elevates a 32K IGFBP (IGFBP-1) and lowers the 38–45K BP bands (IGFBP-3) in the rat. Levels of a 24K IGFBP responded to energy but not protein level in the diet. Furthermore, the presence of adequate energy with protein deficiency resulted in more severe changes in levels of IGFBPs, binding protein activity patterns, and serum IGF-I levels than fasting or re-feeding a diet inadequate in both protein and energy.

Materials and methods

Experimental protocol

Fifty weanling male Sprague-Dawley (100–120 g) rats (Harlan Sprague Dawley, Inc., Madison, WI) were randomly assigned to five treatment groups of 10 animals per group. All animals were fed an adequate control diet (20% protein as egg white solids, 63% carbohydrate, 10% fat, 7% non-caloric constituents, 4.24 kcal/g) ad libitum for a 5-day acclimation period.[23] On day 5 (between 8:00 and 10:00) one group of animals was anesthetized with ether and killed by exsanguination as the baseline group. All remaining rats were fasted for 48 hours with free access to water. On day 7, a second group was killed as above and designated the fasted group. After the fast, the remaining animals were re-fed one of the following diets for a 48-hour period (to day 9):

1. Control (adequate) fed ad libitum;
2. Low protein-low energy: control diet fed at 50% of ad libitum intake during the acclimation period;
3. Low protein-isocaloric: 5% protein, isocaloric to control by adjustment of carbohydrate fed ad libitum.

On day 9 these three groups were killed as above. Blood was allowed to clot for 1 hour on ice, centrifuged (3000*g* for 15 min), aliquoted, and frozen at −20° C until analysis. Body weights and feed intake were measured daily. All animal protocols were conducted according to NIH guidelines. Animal use protocols were approved by The Animal Care and Use Committee of The Ohio State University.

Radioimmunoassays (RIA)

IGF-I levels in serum were measured using a heterologous RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) immediately after acid ethanol extraction. The method of acid ethanol extraction was a modification[24] of previously published procedures.[25] The antibody used in this assay exhibits approximately 2% cross-reactivity with IGF-II and minimal cross-reactivity (< 0.03%) with proinsulin, porcine insulin, or human GH.[26] Recombinant human IGF-I (Imcera Bioproducts, Terre Haute, IN) was used as a standard in the assay. In sera that were acid ethanol extracted, neutralized, incubated with [^{125}I] IGF-I and chromatographed through a Sephadex G-50 column (Pharmacia, Piscataway, NJ) at pH 7.4, at least 89% of the [^{125}I] IGF-I eluted as free peptide, indicating little interaction of the added IGF-I with residual binding proteins. Various dilutions of acid ethanol extracted rat serum exhibited parallelism with the human IGF-I standard. Serum insulin was measured utilizing an insulin RIA kit (Cambridge Medical Diagnostic, Billerica, MA).

Serum IGF binding protein patterns

Serum IGF-I binding protein patterns were obtained using the method of D'Ercole et al.[27] Triplicate pooled serum samples (0.45 mL) from each group were pre-incubated at 4° C for 16–18 hr in the presence of 100,000 dpm [^{125}I] IGF-I (2,000 Ci/mM) (Amersham Corp., Arlington Heights, IL). The serum was then chromatographed on a calibrated 1.6 × 90 cm Sephacryl S-300 (Pharmacia) gel filtration column equilibrated in 20 mM Tris-HCl, pH 6.9, 100 mM NaCl, 0.025% NaN_3. The column was run at 10 mL/hr and fractions were collected every 15 minutes. Non-specific binding (NSB) was determined by chromatographing serum pre-incubated as above with excess unlabeled IGF-I. Fractions were counted on a gamma counter (Isodata model 2020, Isodata, Palatine, IL) to detect bound [^{125}I] IGF-I. Specific binding was determined by subtracting the corresponding NSB from each fraction.

Ligand blotting

IGF-I binding proteins of different molecular weights were measured using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic blotting to nitrocellulose and probing with radiolabeled IGF-I.[28] Quadruplicate samples of the various treatment sera were diluted and mixed with Laemmli's electrophoresis buffer (without beta-mercaptoethanol) such that 2 μL of serum was applied to each well. These samples were subjected to SDS PAGE.[29] Separated protein bands were electrophoretically transferred onto nitrocellulose paper. The paper was quenched by incubating with 1% nonfat dry milk, rinsed, and incubated with the radiolabeled ligand (4000 cpm/cm^2 [^{125}I] IGF-I) for 16 hours at 4° C with or without 100 ng/mL unlabeled IGF-I. The blot was rinsed, dried, and placed in a cassette with Kodak XAR film at −70° C and exposed for 7 days. All bands showing IGF binding were completely removed by incubation with unlabeled IGF-I. The SDS PAGE procedure strips endogenous IGF from the binding proteins leaving binding sites available to bind the labeled

IGF-I.[28] Thus, the amount of bound [^{125}I] IGF-I should be proportional to total IGF binding protein in the different molecular weight bands. Laser densitometry (LKB Ultroscan XL, Pharmacia LKB, Piscataway, NJ) of the autoradiographs was used to quantitate the different IGFBPs. Data reported as 45K are the sum of the signal intensities of all the bands in the 38–50K region. The 32K and 28K bands were very close in size and difficult to resolve. Migration distance and laser densitometry were used to separate these bands. The signal intensity of each specific IGFBP band was proportional to the amount of serum loaded on ligand blots run with various concentrations of serum.

Statistical analysis

Data were analyzed by one-way analysis of variance. Preplanned mean separations were determined with agreement of approximate *t*-test, Bonferroni's, and Scheffe's S method for multiple comparisons.

Results

The level of energy intake (*Table 1*) for rats re-fed the low protein-low energy diet was 66% lower than for animals re-fed low protein-isocaloric or control diets ($P < 0.05$). The animals re-fed low protein-low energy and low protein-isocaloric diets consumed similar amounts of protein, which were approximately 70% lower than control ($P < 0.05$). Energy content of the diet appeared most important for body weight gain after fasting. Animals re-fed the low protein-isocaloric diet gained a moderate amount, while re-fed low protein-low energy rats just maintained fasted body weight.

Fasting caused a 62% decrease in serum IGF-I levels ($P < 0.05$) compared to baseline (*Table 2*). Re-feeding a control diet for 48 hrs after fasting caused serum IGF-I levels to return to 78% of baseline levels ($P < 0.05$). Re-feeding a low protein-low energy diet maintained serum IGF-I near fasted levels (33% below re-fed control, $P < 0.05$). Despite the moderate body weight gain, rats re-fed the low protein-isocaloric diet had serum IGF-I levels 50% below fasted levels ($P < 0.05$). This was 76% lower ($P < 0.05$) than re-fed control levels despite similar energy intake and 64%

Table 1 Protein intake, energy intake and body weight change during re-feeding

Re-fed group	Protein intake g	Energy intake kJ	Weight Change g
Control	6.6 ± 0.4[a]	585 ± 38[a]	24.7 ± 3.7[a]
Low protein-Low energy	2.2 ± 0.1[b]	196 ± 4[b]	−0.8 ± 0.6[b]
Low protein-isocaloric	1.7 ± 0.1[b]	610 ± 21[a]	15.2 ± 0.7[c]

[a,b,c] Values represent the mean ± SEM of eight to ten animals per group. Means within each column with different superscript differ significantly ($P < 0.05$).

Table 2 Serum IGF-I and serum insulin levels

Group	IGF-I ng/ml	Insulin pM
Baseline	400 ± 15.7[a]	161.5 ± 17.4[a]
Fasted	151 ± 7.8[b]	105.6 ± 18.8[a]
Re-fed control	316 ± 16.7[c]	316.0 ± 43.5[b]
Re-fed low protein-low energy	211 ± 17.0[b]	79.3 ± 6.0[a]
Re-fed low protein-isocaloric	76 ± 12.4[d]	111.1 ± 16.1[a]

[a,b,c,d] Values represent the mean ± SEM of eight to ten animals per group. Means within each column with different superscripts differ significantly ($P < 0.05$).

below low protein-low energy ($P < 0.05$) despite equivalent protein restriction.

Serum insulin decreased 35% with fasting (*Table 2*). Insulin levels increased above baseline levels when fasted rats were re-fed a control diet. However, re-feeding low protein-low energy or low protein-isocaloric diets to fasted rats did not raise serum insulin levels.

Figure 1 compares the changes in serum IGF-I binding protein activity patterns in the 155K and 50K regions of the gel filtration column chromatograph. Actual cpm of labeled IGF-I bound in the 155K and 50K regions of the chromatograph are shown in *Table 3*. IGFBP activity in the 150K class of binding protein paralleled serum IGF-I levels. The binding protein activity in the 50K region responded in an opposite manner. Fasting caused a 150% increase in IGF-I binding activity in the 50K region and a 50% decrease in the 155K region. Re-feeding fasted rats a control diet restored both peaks to baseline levels. IGF binding activity of the 155K and 50K regions remained at fasted levels in rats re-fed a low protein-low energy diet. Compared to re-fed control rats (*Table 3*), IGF binding activity in rats re-fed a low protein-isocaloric diet was 188% greater in the 50K region and 67% lower in the 155K region. Energy level with protein restriction was important since responses in both IGF binding peaks were different between low protein-low energy and low protein-isocaloric groups.

Ligand blotting (*Figures 2* and *3*) revealed bands of specific IGF-I binding at molecular weights of 24K, 28K, 32K, and a group of bands ranging from 38–50K (45K group) similar to those previously reported in rats.[13,30,31] Since nutritional deprivation resulted in decreased IGF-I binding in the 45K bands, but increased binding in 32K and some 24K bands, the total amount of IGFBPs was not significantly different among fasted and re-fed groups.

Protein restriction at both energy levels caused the 45K bands (IGFBP-3) to decrease, and the 32K band (IGFBP-1) to increase compared to baseline, fasted, or re-fed control levels. The IGFBP-1 band exhibited a 144% greater signal intensity in re-fed low protein-isocaloric rats and a 111% greater intensity in low protein-low energy rats compared to re-fed controls.

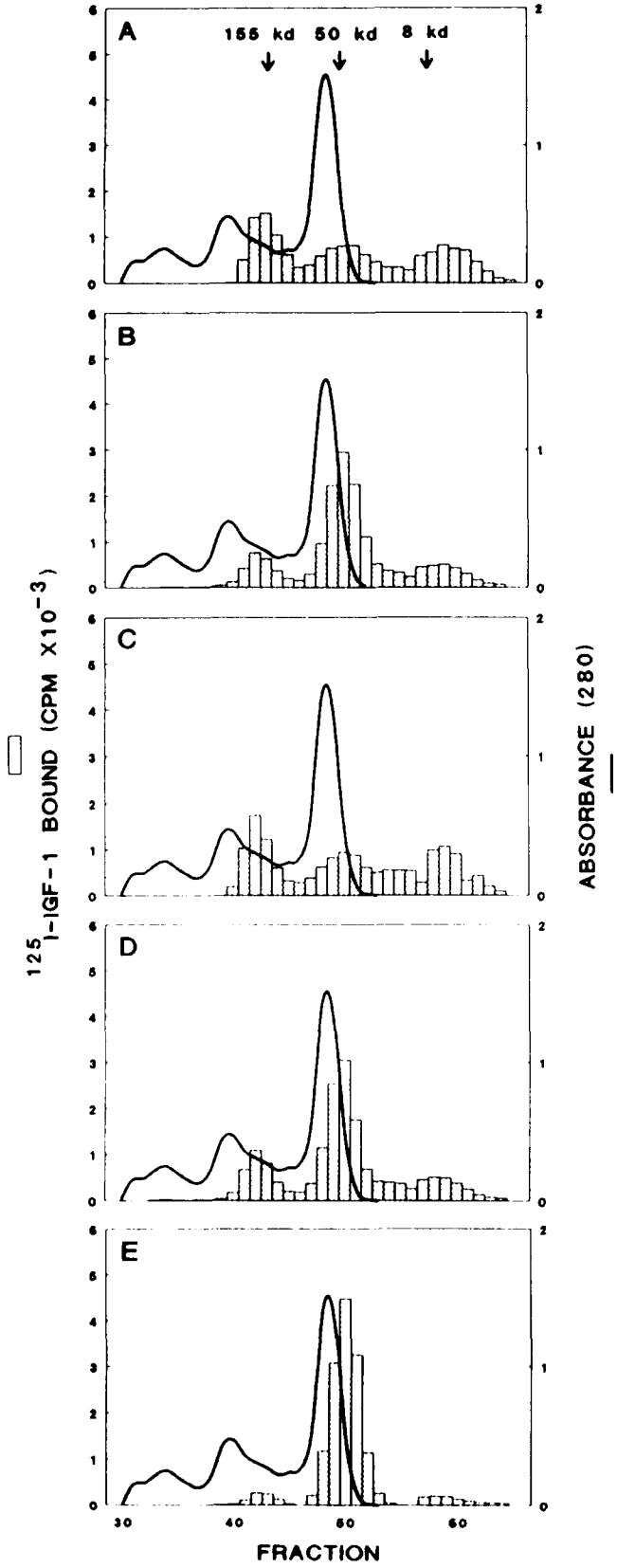

Figure 1 Fractionation of 0.45 mL of (A) baseline serum, (B) fasted serum, (C) re-fed control serum, (D) re-fed low protein-low energy serum, and (E) re-fed low protein-isocaloric (energy adequate) serum on Sephacryl S-300 after a 16 hr pre-incubation with [^{125}I] IGF-I as described in Materials and methods. This figure shows the absorbance at 280 nm (———) of the chromatograph, and the open bars (⊓) indicate the serum IGF-I binding protein activity in the 155K and 50K molecular size ranges. Unbound [^{125}I] IGF-I is shown in the 8K region of the chromatograph. Nonspecific binding of [^{125}I] IGF-I has been subtracted from IGF binding protein activity data.

Table 3 Changes in serum IGF-I binding protein activity patterns

	155K		50K	
	CPM bound [^{125}I] IGF-I	Percent change from control	CPM bound [^{125}I] IGF-I	Percent change from control
Baseline	5,567	−7	4,548	−6
Fasted	3,278	−45[b]	10,568	+118[b]
Re-fed control	6,014		4,838	
Re-fed low protein-low energy	4,117	−32	10,317	+113[b]
Re-fed low protein-isocaloric	2,001	−67[b]	13,933	+188[b]
Pooled SEM[a]	775		1,406	

[a] Pooled standard error of the mean.
[b] Significantly different from Re-fed control, $P < 0.05$.

There was no apparent change in the 28K protein band due to nutritional treatment. The 24K band responded to energy content of the diet, with no significant change observed between control, or low protein-isocaloric diets. When energy was restricted, levels of the 24K protein decreased and were less than rats re-fed low protein-isocaloric diets.

Discussion

Our findings provide new information that demonstrates that serum levels of IGFBP-1, IGFBP-3, and a 24K binding protein and IGF binding activity patterns are very sensitive to the ratio of dietary protein and energy in rats. Our results demonstrate that fasting elevates IGFBP-1 in growing rats similar to what has been shown in humans.[15-17] In addition, re-feeding diets deficient in protein and/or energy results in further elevation of IGFBP-1 above fasted levels. This is consistent with other studies that demonstrate the sensitivity of IGFBP-1 to metabolic status.[14,30,32,33]

Furthermore, our study demonstrates that IGFBP-3 decreases with serum IGF-I levels during protein deprivation, as has been recently shown by Clemmons, et al.[31] However, our data show that IGFBP-1 and the 24K IGFBP are also responsive to nutrition, whereas, these investigators report that only IGFBP-3 was sensitive to dietary protein. This discrepancy may be explained by the acute post-fast deprivation studied in our investigation versus a more chronic deficiency studied by Clemmons et al.[31]

We have found that levels of the 24K IGFBP were decreased with fasting and re-feeding a low protein-low energy diet. Thus, this IGFBP responded to energy but not protein restriction. Other studies have

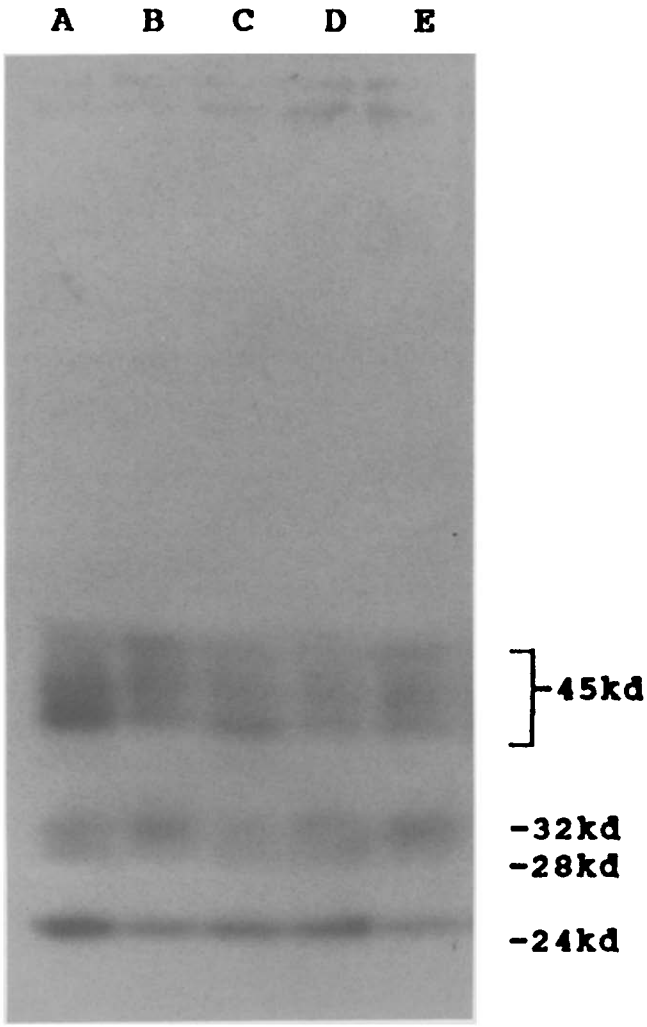

Figure 2 Autoradiograph of a SDS 7.5%-15% gradient polyacrylamide gel of rat serum after ligand blotting with [^{125}I]IGF-I. Serum samples were electrophoresed, transferred to nitrocellulose, and probed as described in **Materials and methods.** Lane A is baseline serum; lane B is fasted serum; lane C is re-fed control serum, lane D is re-fed low protein-low energy serum; lane E is re-fed low protein-isocaloric (energy adequate) serum. The molecular weights of the bands are indicated in kilodaltons (kd). The following prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) were used: myosin heavy chain (200 kd), phosphorylase B (97.4 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), carbonic anhydrase (29kd), beta-lactoglobulin (18.4 kd), and lysozyme (14.3 kd).

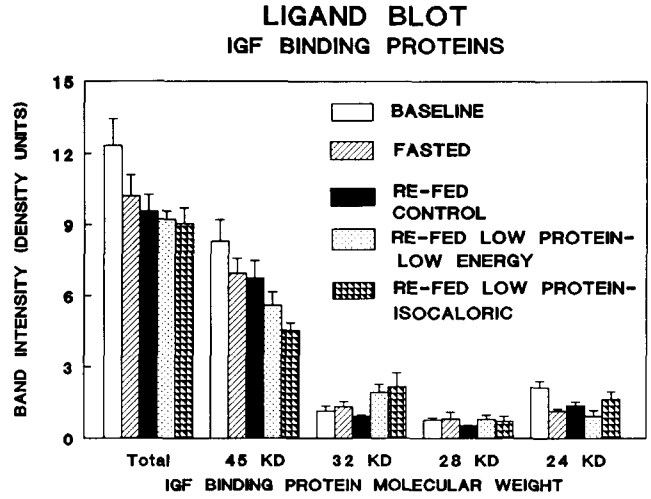

Figure 3 Laser densitometry of ligand blot autoradiographs. Relative changes in the band density are shown for the total sample and the 45 kd, 32 kd, 28 kd, and 24 kd bands of specific IGF binding. Data for each band is graphed by the band's apparent molecular weight and expressed as scanning density units ± SEM.

shown that this protein becomes the predominate IGFBP during pregnancy in rats,[34] however, few other regulatory characteristics of this protein are known.

In adult humans, a very low energy intake results in greater impairment of post-fast recovery of serum IGF-I than an equivalent restriction of protein.[18] In young growing rats, protein appears to be more critical than energy,[2,19] and this effect attenuates with age.[20] Our study points to the importance of the ratio of protein-to-energy in the diet. When total grams of protein consumed per day is inadequate, the presence of adequate energy had a more severe effect on IGFBPs and further decreased serum IGF-I levels. When careful comparison of diets is made, this effect on serum IGF-I levels can be observed in more chronic feeding studies as well.[19]

When both energy and protein intakes are deficient, metabolic adaptations occur that preserve body protein more efficiently, leading to the better metabolically adapted syndrome of marasmus. When normal levels of energy are present with protein deficiency, this adaptation is disrupted resulting in the decreased serum albumin, edema, and fatty liver observed in kwashiorkor.[22] Our study indicates that serum IGF-I, IGF binding activity patterns, and levels of IGFBP-1 and IGFBP-3 are acutely sensitive to these metabolic adaptations.

It has been shown that serum albumin levels and hepatic albumin mRNA levels are decreased in low protein-isocaloric but not low protein-low energy fed rats.[35] This suggests a generalized decrease in hepatic protein synthesis in kwashiorkor-like protein-energy malnutrition. This may explain our observations of decreased serum IGF-I levels and IGFBP-3 levels with low-protein-isocaloric diets, but does not explain the rise observed in IGFBP-1. Clearly, these binding proteins are differentially regulated. The responsiveness of individual IGFBPs to nutritional manipulation, particularly to the ratio of protein to energy, suggests the possibility of using measurements of IGFBPs as markers of acute changes in nutritional and metabolic status.

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