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Reduction of Serum Insulin-like Growth Factor-I by Dietary Protein Restriction Is Age Dependent

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ABSTRACT. We have determined if dietary protein restriction for 1 wk has differential effects on growth, serum IGF-I, and liver growth hormone receptors at various stages of development. Female Wistar rats were fed a low (5%) protein diet for 7 d at 3, 4, 6, 8, and 12 wk of age, whereas controls were maintained on a normal (15%) protein diet. Body wt gain was impaired in the groups fed the low protein diet, despite normal energy intake, and the effect was attenuated with age. Liver cell number (DNA content) was reduced by low protein feeding in the 3-, 4-, and 6-wk age groups ($p < 0.01$), but not in the older animals. Protein restriction caused a dramatic decrease in serum IGF-I in the younger animals (90 and 82% reduction *versus* normal fed age-matched controls, at 3 and 4 wk, respectively; $p < 0.001$), and this effect was progressively attenuated with increasing age (49, 40, and 25% reductions of serum IGF-I at 6, 8, and 12 wk, respectively). Changes in serum IGF-I correlated with those of liver cell number ($r = 0.80$; $p < 0.001$). Total and free liver growth hormone receptors were slightly decreased in the low protein diet groups at 4 ($p < 0.05$) and 6 wk (total: $p < 0.001$; free: $p < 0.01$) but not in the other age groups. The occurrence of profound diet induced reductions in IGF-I without proportional reductions in liver GH receptors suggest that the apparent GH resistance occurs at a postreceptor level. Because the degree of IGF-I reduction correlates with the severity of retardation of liver growth during dietary protein restriction at different ages, we conclude that the effect of protein restriction on liver growth could be mediated through IGF-I. (*Pediatr Res* 26: 415–419, 1989)

Abbreviations

rGH, rat growth hormone
bGH, bovine growth hormone
ANOVA, analysis of variance

The GH-dependent peptide IGF-I is widely believed to be one of the principal stimulators of the cell proliferation that results in somatic growth (1). Regulated by food intake and nutritional status (2, 3), serum concentrations of IGF-I are reduced by restriction of energy or protein (4–6). These reductions in IGF-I, which occur despite adequate serum GH concentrations, may be due to decreased liver GH receptors or postreceptor defects in GH action (4, 7). The adverse effects of nutrient deprivation on growth are greatest in young animals, in whom growth depends heavily on cell proliferation (8, 9). The study reported here was undertaken to determine whether dietary protein restriction in rats at various times between weaning and postpuberty (3–12 wk of age) has differential effects on serum IGF-I concentrations. Specifically, we hypothesized that young animals, in whom malnutrition impairs growth most severely, would experience the greatest reductions in serum IGF-I. To determine the mechanisms that might regulate IGF-I in such animals, we also have determined serum concentrations of GH and insulin, as well as liver GH binding.

MATERIALS AND METHODS

Animals and experimental design. Female Wistar rats were housed individually in metabolic cages under controlled conditions (light from 0600–1800 h; constant temperature, 22°C) and fed a normal protein powdered diet (15% casein; 325 cal/100 g). At the ages of 3, 4, 6, 8 and 12 weeks, 6 rats in each age-group (12 rats/group) were maintained on the normal protein diet for 1 additional wk, and six rats were fed a low protein isocaloric diet (5% casein; 325 cal/100 g) for the same period. Body wt and food intake were recorded daily, and after 7 d the animals were killed by decapitation between 0930 and 1100 h. Blood was collected from the trunk vessels and kept at 4°C for up to 60 min. After centrifugation, the serum was stored at −20°C until assayed for IGF-I, rGH, insulin, and glucose. At death, each liver was removed promptly, weighed, homogenized as previously described (10), and stored at −20°C until assayed for bGH binding, protein, and DNA content.

RIA and binding studies. IGF-I was measured by RIA on unextracted serum using a nonequilibrium technique (11, 12). A pool of sera from 10 adult male rats was used as the standard; its potency was designated as 1 U/mL. To insure that differences in the IGF-I measured between experimental groups were not due to interference of IGF-I binding proteins in the RIA, IGF-I was also assayed after extraction on pools of equal volumes of serum samples from each experimental group ($n = 16$). These pools of sera were acidified and extracted using ODC-silica columns (C18 Sep-Pak; Waters Associates, Milford, MA), sequentially eluting binding proteins with 7% acetic acid and IGF-I with 100% methanol (13, 14). With this technique, >99% of binding proteins are removed and 68% of IGF-I present in serum is recovered. In assays of extracted serum, pure plasma-derived IGF-I (PS III) was used as standard and corrections are made for losses during extraction. Serum rGH was measured (14) using

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the reagents prepared by Dr. A. F. Parlow (UCLA, Harbor Campus, Torrence, CA) and obtained through the NIDDK Rat Pituitary Program (National Institute of Diabetes, Digestive Diseases and Kidney, National Institutes of Health, Bethesda, MD). Serum insulin concentrations were determined by RIA as reported previously (15), except that the second antibody was a solid-phase coupled antiguinea pig γ-globulin (IRE, Fleurus, Belgium). Glucose was measured in serum by an automated glucose-oxidase method (Beckman Instruments, Fullerton, CA).

Somatogenic binding sites were determined on liver homogenates using ^{125}I-bGH (10). The highly purified bGH used for iodination was generously provided by Dr. A. C. Paladini (Buenos-Aires, Argentina). Bovine GH (USDA B-1) was used as unlabeled hormone for the determination of nonspecific binding. Specific binding of bGH was measured by determining the difference between the radioactivity bound in the absence and in the presence of unlabelled hormone (1 μg/tube). To measure total bGH binding, liver homogenates were treated for 10 min with 4 M $MgCl_2$ at room temperature to remove endogenous ligand (10, 16). Tracer binding determined after $MgCl_2$ treatment were corrected for protein loss (22.4 ± 5.8% *versus* H_2O-treated homogenates; mean ± SD). Free receptors were assessed simultaneously on water-treated homogenates. Proteins were determined by the method of Lowry *et al.* (17), and DNA was measured by fluorometry, as described by Karsten and Wollenberger (18).

Statistical analysis. Data were analyzed by two-way ANOVA using the Statistical Analysis System (19) to determine the respective influences of the two main factors (protein content of the diet and age of the rats) and their interactions on the different variables studied. The variables are shown as mean ± SEM. When the diet effect was significant, or when interaction between the two factors was significant ($p < 0.05$) for one variable differences between means were analyzed by t tests using the pooled residual mean square of the ANOVA, to determine the specific effect of diet at each age separately.

RESULTS

Body wt gain was impaired during protein restriction in the 3 to 8 wk age groups ($p < 0.001$) but not in the 12 wk age group ($p > 0.05$) (Table 1). The effect was age-dependent (age × diet interaction in ANOVA for body weight gain: $p < 0.001$), being progressively less pronounced as the animals increased in age. Similarly, a slight but significant effect of protein restriction on growth of the tail was observed in the 4- and 6-wk age groups. DNA content per liver, an index of cell number, was reduced significantly by the protein restriction in the 3-, 4-, and 6-wk groups (35, 28, and 20%, respectively; $p < 0.01$), but not in the 8- and 12-wk groups. Liver protein content and cell size (protein/DNA ratio) were lower in each of the malnourished groups, regardless of age (Table 2).

Table 1. *Food intake, body wt gain, and tail length in rats fed normal and low protein diet (mean ± SEM)*

Age group (wk)*	Diet (% protein)	Food intake (g/d)	Body wt gain (g)†	Tail length at sacrifice (cm)
3	15	10.4 ± 0.3	28.6 ± 1.1	9.5 ± 0.3
	5	9.6 ± 0.5	7.3 ± 0.7‡	8.9 ± 0.2
4	15	12.0 ± 0.6	33.8 ± 1.2	10.5 ± 0.3
	5	11.6 ± 0.3	10.1 ± 1.3‡	9.8 ± 0.4§
6	15	15.9 ± 0.2	20.6 ± 1.4	14.4 ± 0.4
	5	16.7 ± 0.7	3.7 ± 1.6‡	13.4 ± 0.4‖
8	15	15.3 ± 0.7	18.7 ± 2.0	15.6 ± 0.3
	5	16.6 ± 0.8	8.4 ± 2.1‡	16.0 ± 0.3
12	15	17.3 ± 0.8	8.4 ± 1.4	17.6 ± 0.3
	5	17.7 ± 0.5	5.6 ± 1.3	17.6 ± 0.3

* Age at which the 1-wk experimental period is begun.
† Body wt gain over the 1-wk experimental period.
‡ $p < 0.001$ *versus* 15% protein diet.
§ $p < 0.05$ *versus* 15% protein diet.
‖ $p < 0.01$ *versus* 15% protein diet.

Before puberty (3 and 4 wk) the serum IGF-I concentration was profoundly reduced by protein restriction, being 0.5 and 0.6 U/mL in control rats and 0.05 and 0.1 U/mL in the 5% protein-fed rats, respectively (90 and 83% decrease in comparison to the age-matched controls; $p < 0.001$; Fig. 1). At later stages of development, IGF-I was reduced less dramatically by protein deprivation. Thus at 6, 8, and 12 wk of age, IGF-I was reduced by 49% ($p < 0.001$), 40% ($p < 0.001$), and 25% ($p < 0.05$) in comparison to the age-matched controls (Fig. 1). This age dependency of diet-induced reductions in IGF-I was highly significant (age × diet interaction in ANOVA for IGF-I: $p < 0.001$).

The percent decrease of serum IGF-I after protein restriction was slightly less in all age groups when assayed after extraction than when determined in unextracted samples (Table 3). However, the age effect of the IGF-I reduction induced by the low protein diet remained evident after sample extraction, because the IGF-I decrease was 69% at 4 wk and only 17% at 12 wk. Diet-induced changes of IGF-I measured on unextracted sera correlated significantly with liver DNA content ($r = 0.80$; $p < 0.001$; Fig. 2), but not with protein/DNA ratios ($r = 0.24$; $p > 0.05$).

The low protein diet did not change serum GH concentrations but caused a significant mean 43% decrease in serum insulin, which was not age-dependent (overall diet effect in ANOVA: $p < 0.001$; comparison of differences between means: $p < 0.05$ at wk 4, 8, and 12). Serum glucose did not change with age or diet (Table 4).

Compared to controls, 7 d of protein restriction caused total ($MgCl_2$-treated homogenates) liver GH binding sites to be reduced by 38% ($p < 0.05$) and 45% ($p < 0.001$) in the 4- and 6-wk age groups, respectively (Fig. 3). No change was observed in the 3-wk or in the 8- and 12-wk age groups. Total liver GH binding sites increased with age in control animals, the most significant rise occurring at the onset of puberty (between 4 and 6 wk of age). The changes in free (H_2O-treated homogenates) liver binding sites paralleled those of total GH receptors.

DISCUSSION

Our study shows that the serum IGF-I concentrations are critically dependent on dietary protein supply at the early stages of development, and that this protein dependence decreases with age. One wk of protein deprivation reduced IGF-I concentrations in prepubertal rats by 90%, the values being as low as those found in hypophysectomized animals. This response to the nutritional insult became progressively less pronounced with increasing age, so that protein deprivation in postpubertal rats caused only a 25% reduction in IGF-I. The changes in IGF-I observed are attributable primarily to the dietary protein deprivation, because the energy intakes of protein-restricted rats were equivalent to controls. Our observations agree with the results of Prewitt *et al.* (6), which show that serum IGF-I in postweanling rats is influenced by dietary protein and that reduction of IGF-I has a linear relationship to the degree of protein restriction. The results obtained are not due to nonspecific interference by IGF binding proteins in the RIA on unextracted serum, because similar changes were also observed when IGF-I was determined after separation from the binding proteins. The results, however, do not exclude the possibility that the changes in IGF-I observed

Table 2. *Liver wt, DNA, and protein contents, and protein DNA/ratios in rats fed normal and low protein diets (mean ± SEM)*

Age groups (wk)*	Diet (% protein)	Liver wt (g)	DNA (mg/liver)	Protein (mg/liver)	Protein DNA
3	15	3.1 ± 0.2	12.0 ± 0.6	485 ± 22	41 ± 1
	5	1.9 ± 0.1†	7.8 ± 0.6‡	238 ± 13†	31 ± 2‡
4	15	3.5 ± 0.1	13.8 ± 1.2	547 ± 27	41 ± 3
	5	2.6 ± 0.2‡	10.0 ± 0.9‡	320 ± 18†	33 ± 2§
6	15	5.5 ± 0.2	19.3 ± 0.3	848 ± 56	44 ± 4
	5	5.1 ± 0.3	15.5 ± 0.7‡	664 ± 27‡	43 ± 1
8	15	6.3 ± 0.4	21.3 ± 0.7	1015 ± 63	48 ± 3
	5	6.2 ± 0.4	22.3 ± 0.8	860 ± 72§	40 ± 3§
12	15	6.7 ± 0.1	22.7 ± 0.8	1099 ± 40	49 ± 3
	5	6.7 ± 0.3	23.3 ± 1.4	920 ± 46‡	40 ± 2§

* Age at which the 1-wk experimental period is begun.
† $p < 0.001$ *versus* 15% protein.
‡ $p < 0.01$ *versus* 15% protein.
§ $p < 0.05$ *versus* 15% protein.

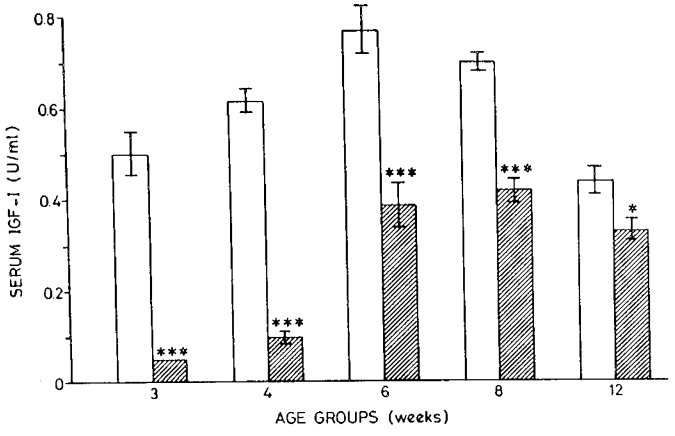

Fig. 1. Effect of 1-wk dietary protein restriction upon serum IGF-I at different ages. The groups fed the low and normal protein diets are represented by the hatched and open bars respectively. The various age groups are as represented. Data are mean ± SE. *, ***: $p < 0.05$ and < 0.001 *versus* normal fed controls of the same age.

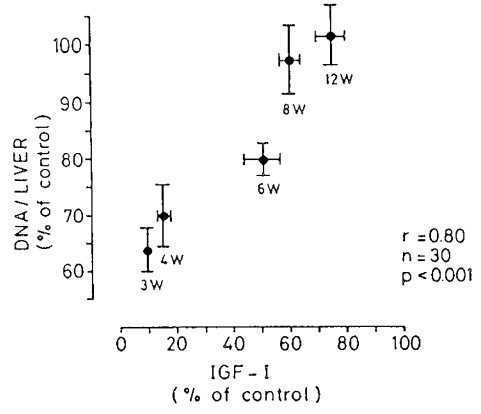

Fig. 2. Comparison of the effects of 1-wk dietary protein restriction upon serum IGF-I (unextracted) and liver DNA content at various ages. The IGF-I and DNA/liver in the protein restricted rats of each age group are expressed as a percentage of the values determined in the normal fed controls of the same age (percent of controls). The data are represented as means of each age group ± SE.

Table 3. *Serum IGF-I after extraction in rats fed normal and low protein diet*

Age group (wk)*	Diet (% protein)	IGF-I (extracted)† 5% *vs* 15% protein diet (nmol/L)	(% decrease)‡
3	15	136	66
	5	46	
4	15	169	69
	5	53	
6	15	244	29
	5	173	
8	15	286	21
	5	226	
12	15	230	17
	5	190	

* Age at which the experimental period is begun.
† Equal volumes of serum from each experimental group were pooled, extracted and assayed for IGF-I as described in "Materials and Methods."
‡ 15% protein − 5% protein/15% protein × 100.

Table 4. *Serum glucose, insulin, and growth hormone in rats fed the normal and low protein diet*

Age groups (wk)*	Diet (% protein)	Serum glucose (mmol/L)	Serum insulin (pmol/L)†	Serum growth hormone (ng/mL)‡
3	15	7.8 ± 0.2	145 ± 14	8 (5–129)
	5	8.1 ± 0.6	53 ± 3	12 (<5–39)
4	15	8.1 ± 0.3	205 ± 51	11 (8–16)
	5	7.7 ± 0.3	79 ± 15§	7 (<5–25)
6	15	7.9 ± 0.3	267 ± 55	17 (6–212)
	5	7.7 ± 0.2	210 ± 70	9 (<5–17)
8	15	7.9 ± 0.3	424 ± 56	48 (15–213)
	5	7.9 ± 0.3	284 ± 22§	28 (9–131)
12	15	7.4 ± 0.4	374 ± 44	68 (17–273)
	5	7.3 ± 0.2	236 ± 36§	76 (23–149)

* Age at which the 1-wk experimental period is begun.
† Determined at time of death; mean ± SE.
‡ Determined at time of sacrifice; median with range; results are expressed as ng/mL of the rGH-RP-1 reference preparation.
§ $p < 0.05$ *versus* 15% protein.

(whether measured on extracted or unextracted samples) are secondary to changes in concentration of IGF binding proteins.

The high degree of sensitivity of the immature rat to protein deprivation was not due to a selective loss of liver GH receptors.

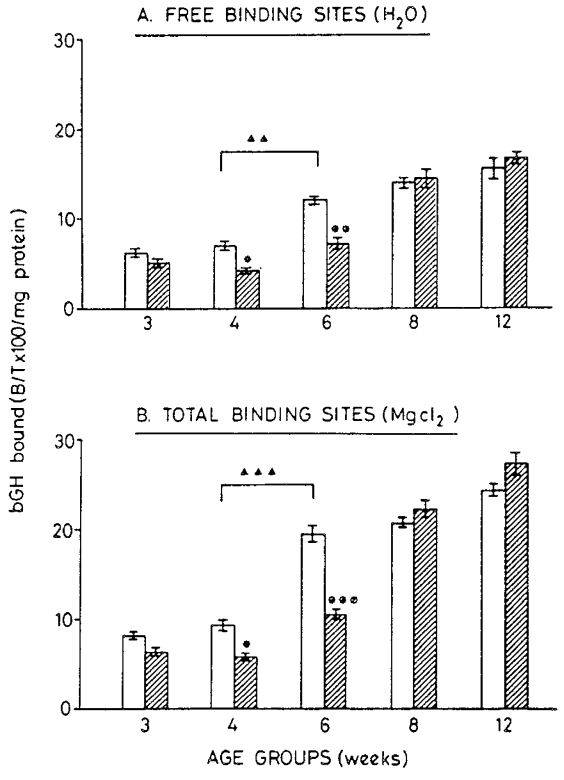

Fig. 3. Effect of 1-wk dietary protein restriction upon liver GH receptors at different ages. The groups fed the low and normal protein diets are represented by the *hatched* and *open bars*, respectively. The free and total binding sites are determined as described in "*Materials and Methods.*" The specifically bound bGH (*B*) is expressed as a percentage of total (*T*) labeled bGH incubated with the liver homogenates. The various age groups are represented. Data are mean ± S.E. *, **, ***: $p < 0.05$, < 0.01, < 0.001 *versus* normal fed controls of the same age. ▲▲, ▲▲▲: $p < 0.01$, < 0.001; 4- *versus* 6-wk age groups.

Indeed, the pronounced decrease in IGF-I observed prepubertally occurred without significant (3 wk) or with minimal (4 wk) changes of such receptors. Furthermore, we still observed significant reductions in IGF-I with protein restriction at later stages of development (8 and 12 wk) without any change in total or free GH binding. These observations tend to support our earlier studies (7, 20) showing that a postreceptor defect(s) plays a predominant role in the induction of GH resistance in dietary protein restriction. In the pregnant rat, dietary protein restriction may have different consequences. Indeed, Pilistine *et al.* (21) showed that in that situation, human GH infusion prevented the serum IGF-I fall but not the body wt loss caused by protein depletion.

Although insulin participates to the maintenance of serum IGF-I concentrations (2) and the protein-restricted animals were hypoinsulinemic, reduced insulin probably does not by itself explain the reduction in IGF-I concentrations. We reach this conclusion because the hypoinsulinemia was not age dependent as were the reductions of IGF-I concentrations. Furthermore, we have shown that protein restriction reduced IGF-I independent of insulin (14).

Before weaning, malnutrition retards cell multiplication (reduction in DNA content per organ), resulting in a permanent stunting of body and organ mass even after prolonged rehabilitation (9, 22–24). In contrast, the predominant effect of malnutrition during early adulthood is to reduce cell size. This allows catch-up growth after refeeding. In this study, we confirm the age dependence of the effects of malnutrition upon liver cell number, and show that this cell deficit correlates with decreased serum IGF-I. The decrease in hepatic mass may contribute to the reduction in plasma IGF-I. At the earlier stages of development, however, 26–39% decrease in liver wt, or 28–35% reduction in liver DNA content (an index of cell number) cannot account for the 83–90% reduction in IGF-I. This suggests that the IGF-I production per cell is reduced. It is possible therefore that the profound reduction in IGF-I caused by protein malnutrition in early development could play a role in the reduced cell number. Although low concentrations of IGF-I in serum may not have caused attenuation of the increase in liver cell number directly, it is most likely that serum concentrations of this growth factor reflect tissue concentrations (25). Because the IGF-I in tissues may act by autocrine or paracrine mechanisms (26), decreased hepatic IGF-I could be a means whereby liver growth is attenuated. Similarly, decreased IGF-I in other tissues during protein deprivation could serve as the mechanism for generalized growth retardation.

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