

# Failure of exogenous IGF-I to restore normal growth in rats submitted to dietary zinc deprivation

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## Abstract

Dietary zinc deficiency in rats causes growth retardation associated with decreased circulating IGF-I concentrations. To investigate the potential role of low IGF-I in this condition, we attempted to reverse the growth failure by administration of exogenous IGF-I. Rats were fed for 4 weeks a zinc-deficient diet (ZD, Zn 0 ppm) or were pair-fed a zinc-normal diet (PF, Zn 75 ppm). We compared the anabolic action of recombinant human (rh) IGF-I infused at the dose of 120 µg/day for the last experimental week in ZD, PF and freely fed control (CTRL) rats. Zinc deficiency caused growth stunting (weight gain 47% of PF;  $P < 0.001$ ), decreased circulating IGF-I (52% of PF;  $P < 0.01$ ) and liver IGF-I mRNA (67% of PF;  $P < 0.01$ ). Serum insulin-like growth factor-binding protein-3 (IGFBP-3) assessed by ligand blot was also

reduced in ZD rats (65% of PF;  $P < 0.01$ ). While exogenous IGF-I increased body weight in CTRL (+12 g;  $P < 0.01$ ) and PF (+7 g; not significant) animals, growth was not stimulated in ZD rats (−1.5 g) in comparison with the corresponding untreated groups. However, circulating IGF-I and IGFBP-3 levels were restored by IGF-I infusion to levels similar to those in untreated CTRL rats. In conclusion, restoration of normal circulating levels of IGF-I and IGFBP-3 by rhIGF-I infusion fails to reverse the growth retardation induced by zinc deficiency. These results suggest that growth retardation related to zinc deficiency is not only caused by low serum IGF-I concentrations, but also by inhibition of the anabolic actions of IGF-I.

*Journal of Endocrinology* (1998) **159**, 211–217

## Introduction

Insulin-like growth factor-I (IGF-I) mediates most of the post-natal growth-promoting actions of growth hormone (GH). Besides GH (Gronowski & Rotwein 1995), insulin (Maes *et al.* 1983) and protein intake (Thissen *et al.* 1994), micronutrients like zinc have also been shown to control IGF-I synthesis and its release into the circulation (Ninh *et al.* 1995). Indeed, in rats, as in humans, dietary zinc deprivation is associated with low serum IGF-I concentrations. Previous experiments of our group have shown that the decline in IGF-I caused by zinc deprivation results from a state of GH resistance (Ninh *et al.* 1997).

Reduced circulating IGF-I levels have been proposed as a potential mechanism for the growth retardation induced by zinc deficiency (Cossack 1984, Oner *et al.* 1984). This hypothesis is supported by several observations showing that zinc deficiency rapidly induces several changes in the somatotrope axis leading to decreased IGF-I (Bolze *et al.* 1987, Dorup *et al.* 1991). However, given its role in the regulation of several enzymes involved in cell proliferation (e.g. thymidine kinase and RNA polymerase), zinc might also control growth independently of changes in circulating IGF-I.

The goal of this study was therefore to investigate whether restoring circulating IGF-I concentrations to normal, despite the persistence of zinc deficiency, would allow resumption of normal growth rates. Therefore, zinc-deprived rats were continuously infused with exogenous IGF-I in an attempt to reverse the decline of IGF-I and the growth retardation.

## Materials and Methods

### Experimental design

Three-week-old female Wistar rats ( $n=36$ ) were housed in individual metabolic cages with stainless steel frames. The animals were kept under controlled conditions of temperature ( $22 \pm 2$  °C), humidity (53%) and lighting (12 h light: 12 h darkness). After adaptation to metabolic cages and to a powdered zinc-normal diet (Zn 75 ppm) for 7 days, the rats were randomly distributed into three dietary groups (12 rats/group): (1) rats given a freely available zinc-normal diet (CTRL group); (2) rats given a freely available zinc-deficient diet (Zn 0 ppm) (ZD); and (3) rats serving as pair-fed animals and receiving a zinc-normal

diet in the same absolute amount as that consumed the day before by the ZD group (PF). The experimental period lasted 4 weeks, and in each dietary group, six rats received a continuous infusion of 120 µg IGF-I/day by means of an s.c. osmotic minipump during the last week of the experimental period (CTRL+IGF-I, PF+IGF-I, ZD+IGF-I) while the six other rats received saline (CTRL, PF, ZD). The semi-synthetic zinc-normal and zinc-free fodders were obtained through the UAR company (Villemoisson-sur-Orge, France). The ZD diet had an energy content of 1.33 MJ (318 kcal)/100 g and contained (per 100 g) 20 g protein, 6 g fat, 60 g dextrose, 6 g cellulose, 1 g vitamin mixture and 7 g inorganic salts without zinc. This diet was supplemented with ZnSO<sub>4</sub>·H<sub>2</sub>O (206 mg/kg powdered diet) to obtain a zinc-normal diet (Zn 75 ppm). The animals had free access to deionized water throughout the experimental period.

Recombinant human (rh) IGF-I (5 mg/ml) used for treatment was kindly provided by Pharmacia & Upjohn (Stockholm, Sweden). The osmotic minipumps (Model 2001, Alza Corporation, Palo Alto, CA, USA) were implanted on the back of the rats under light ether anesthesia.

Body weight and food consumption were recorded each morning. After the 4-week experimental period, the animals were killed by decapitation between 1100 and 1200 h. Blood was collected from trunk vessels into glass tubes, allowed to clot at 4 °C for at least 1 h and centrifuged at 1000 g for 15 min at 4 °C. The sera were stored at -20 °C until assayed for hormones, binding proteins and glycemia. Livers were flash-frozen in liquid nitrogen and stored at -70 °C until assayed for IGF-I and insulin-like growth factor-binding protein-3 (IGFBP-3) mRNAs. The protocol of this study was approved by the Institutional Animal Care and Use Committee of the University of Louvain at Brussels.

#### RIAs

Serum IGF-I concentrations were measured by RIA on serum samples after removal of the binding proteins by chromatography on Sep-Pak C18 cartridges (Waters, Milford, MA, USA) (Davenport *et al.* 1988). Rat GH and insulin were measured by RIAs, using previously described methods (Maiter *et al.* 1989).

#### Glycemia determination

Serum glucose was measured by the glucose oxidase method (Beckman Gluco-analyzer 2, Palo Alto, CA, USA).

#### Measurement of serum IGFBPs

Serum IGFBPs levels were assessed by ligand blotting (Clemmons *et al.* 1989). Serum samples were size-

fractionated by SDS-PAGE in a Laemmli buffer system under nonreducing conditions, electroblotted onto nylon filters, then probed with 10<sup>6</sup> c.p.m./ml (total vol=4 ml) <sup>125</sup>I-rhIGF-II kindly provided by Eli Lilly (Indianapolis, IN, USA) and visualized by autoradiography at -70 °C. The autoradiograms were scanned using an Ultrascan XL laser densitometry (LKB-Bromma, Bromma, Sweden) and the results are expressed as arbitrary scanning densitometric units. For each sample, density signals were determined for the 45–39 kDa (various glycosylated species of IGFBP-3), the 34–29 kDa (IGFBP-1 and -2 essentially), and the 24 kDa (most likely IGFBP-4) bands. The 34–29 kDa bands were analyzed together because they could not be reliably separated for quantification by scanning densitometry. The abundance of IGFBPs in a pool of sera from normal rats served as control. The size of radioactive protein bands was estimated by comparison to standards from Bethesda Research Laboratories (Gaithersburg, MD, USA).

#### Northern blot hybridization

Total RNA was isolated from individual liver tissues (four rats per group) by the guanidinium thiocyanate/cesium chloride method (Chirgwin *et al.* 1977). Total RNA (20 µg/lane) samples were size separated on a formaldehyde/agarose gel and transferred to Hybond membranes (Amersham, Bucks, UK) by overnight vacuum blotting (Vacugene, Pharmacia, Uppsala, Sweden). After UV cross-linking, the membranes were sequentially hybridized to <sup>32</sup>P-labeled cRNA probes specific for rat IGF-I and IGFBP-3 mRNAs, using previously reported methods (Thissen *et al.* 1991a).

The IGF-I cRNA probe was transcribed from the 194 bp AvaII-HinI rat IGF-I exon 4 cDNA fragment inserted into the plasmid vector Bluescript, using T7 RNA polymerase (Life Technologies, Merelbeke, Belgium). The IGFBP-3 riboprobe was transcribed from the 452 bp HindIII-BamHI rat IGFBP-3 cDNA fragment inserted into the plasmid vector Bluescript, using T3 RNA polymerase. Specific activity of these probes was 10<sup>9</sup> c.p.m./µg. Hybridization with a human <sup>32</sup>P-labeled rRNA 18S antisense (Oncogene Science, Inc., NY, USA) was subsequently performed to estimate the variation in RNA loading. Blots were exposed to X-ray films (X-Omat AR, Kodak, NY, USA) for 12 to 72 h at -70 °C. The amount of hybridized mRNA was quantified by scanning of autoradiograms using an Ultrascan XL laser densitometry scanner (LKB-Bromma).

#### Statistical analysis

Data were analyzed by one-way ANOVA followed by Student–Newman–Keuls test to compare multiple means. In the presence of significant heterogeneity of variance

**Table 1** Effect of a 4-week zinc deficiency on body weight gain and food intake. Data are expressed as means  $\pm$  S.E.M. ( $n=6$ /group)

	CTRL	PF	ZD
Body weight gain (g)	56.4 $\pm$ 2.3	38.3 $\pm$ 2.5 <sup>+++</sup>	17.8 $\pm$ 2.9 <sup>+++</sup> , <sup>SSS</sup>
Food intake (g/day)	10.6 $\pm$ 0.2	8.4 $\pm$ 0.4 <sup>+++</sup>	8.3 $\pm$ 0.4 <sup>+++</sup>

<sup>+++</sup> $P<0.001$  vs CTRL and <sup>SSS</sup> $P<0.001$  vs PF.

(GH and insulin), the data were log-transformed before analysis. Results are presented as the mean  $\pm$  S.E.M.

## Results

### Effects of a 4-week zinc deficiency

Confirming our previous report (Ninh *et al.* 1995), zinc deficiency decreased food intake ( $-22\%$ ,  $P<0.001$ ) and body weight gain ( $-68\%$ ,  $P<0.001$ ) compared with the CTRL animals (Table 1). Body weight gain during the last week was only  $1 \pm 3$  g in ZD rats compared with  $7 \pm 2$  g in CTRL animals (Table 2). By comparison with PF rats, who ate similar amounts of food, ZD rats had lower body weight gain ( $-53\%$ ,  $P<0.001$ ), thymus weight ( $-32\%$ ,  $P<0.01$ ), spleen weight ( $-33\%$ ,  $P<0.05$ ) and kidney weight ( $-18\%$ ,  $P<0.01$ ), thus indicating a specific deleterious effect of zinc deficiency on growth (Table 2).

Rat GH concentrations were decreased in both PF ( $-68\%$ ,  $P<0.05$ ) and ZD ( $-76\%$ ,  $P<0.01$ ) in comparison with CTRL rats (Table 3). Glycemia was similar in all groups and insulin concentrations were reduced by 50%, albeit not significantly, in ZD rats by comparison with CTRL animals (Table 3).

By comparison with the PF animals, after 4 weeks zinc deficiency caused reductions in serum IGF-I concentrations ( $-48\%$ ,  $P<0.01$ ; Fig. 1A) and in liver IGF-I mRNA levels ( $-33\%$ ,  $P<0.01$ ; Figs 1B and 2). The serum levels of IGF-BP-3 were significantly lower in the ZD rats than in the CTRL animals ( $-63\%$ ,  $P<0.01$ ), but not compared with PF rats ( $-35\%$ , NS; Fig. 3A). Other IGF binding proteins were not significantly affected by

any dietary manipulation (Fig. 4). Liver IGF-BP-3 mRNA levels were similar among the three untreated dietary groups (Figs 2 and 3B).

### Effects of IGF-I administration during the last week

Continuous infusion of IGF-I for the last week of the experimental period induced a marked stimulation of body weight gain in CTRL ( $+12$  g,  $P<0.01$  vs untreated CTRL), and a less important, not significant effect in PF rats ( $+7$  g, NS vs untreated PF) (Table 2). In contrast, ZD rats did not grow, despite IGF-I treatment. While the weight of most organs was increased in IGF-I-treated CTRL, only the spleen weight was increased by IGF-I infusion in PF and ZD rats. ( $+50\%$ ,  $P<0.05$ ) (Table 2). IGF-I infusion did not significantly decrease serum insulin, except in ZD rats, but decreased endogenous rat GH levels in CTRL and ZD rats (Table 3). Glycemia (Table 3) and food intake (data not shown) were not affected by IGF-I infusion.

IGF-I administration doubled the serum IGF-I concentrations in the three groups of rats (CTRL:  $+92\%$ ,  $P<0.01$ ; PF:  $+96\%$ ,  $P<0.01$ ; ZD:  $+93\%$ ,  $P<0.001$ , Fig. 1A). However, although the amount of infused IGF-I was identical for all rats, circulating IGF-I concentrations in IGF-I-treated ZD rats were only half those obtained in the two other treated groups, albeit normalized by comparison with untreated CTRL rats. A decrease in liver IGF-I mRNA levels was observed in all IGF-I-treated animals ( $-40\%$  in CTRL,  $-38\%$  in PF,  $-45\%$  in ZD,  $P<0.001$ ) (Figs 1B and 2).

Serum IGF-BP-3 and its liver mRNA levels were significantly increased (two-fold) in response to IGF-I

**Table 2** Effect of a 4-week zinc deficiency and IGF-I infusion during the last experimental week on body and organ weight (g). Data are expressed as means  $\pm$  S.E.M. ( $n=6$ /group)

	CTRL	CTRL+IGF-I	PF	PF+IGF-I	ZD	ZD+IGF-I
<b>Body weight gain</b> (last week)	6.5 $\pm$ 1.7	18.3 $\pm$ 1.1 <sup>**</sup>	3.3 $\pm$ 1.8	10.6 $\pm$ 3.5	1.2 $\pm$ 3.3 <sup>+++</sup>	-0.3 $\pm$ 2.3
Final body weight	128 $\pm$ 2	138 $\pm$ 3 <sup>*</sup>	110 $\pm$ 4 <sup>+++</sup>	114 $\pm$ 4	90 $\pm$ 2 <sup>+++</sup> , <sup>SSS</sup>	92 $\pm$ 3
Thymus	0.39 $\pm$ 0.01	0.42 $\pm$ 0.02	0.28 $\pm$ 0.02 <sup>++</sup>	0.29 $\pm$ 0.04	0.19 $\pm$ 0.02 <sup>+++</sup> , <sup>SS</sup>	0.15 $\pm$ 0.01
Liver	4.80 $\pm$ 0.19	5.62 $\pm$ 0.22 <sup>*</sup>	3.84 $\pm$ 0.26 <sup>+</sup>	3.91 $\pm$ 0.40	3.12 $\pm$ 0.13 <sup>+++</sup>	2.93 $\pm$ 0.18
Spleen	0.42 $\pm$ 0.02	0.51 $\pm$ 0.04 <sup>*</sup>	0.33 $\pm$ 0.02 <sup>+</sup>	0.52 $\pm$ 0.04 <sup>**</sup>	0.22 $\pm$ 0.01 <sup>+++</sup> , <sup>S</sup>	0.33 $\pm$ 0.15 <sup>**</sup>
Kidneys	1.13 $\pm$ 0.02	1.34 $\pm$ 0.02 <sup>***</sup>	1.12 $\pm$ 0.07	1.12 $\pm$ 0.04	0.93 $\pm$ 0.02 <sup>+++</sup> , <sup>SS</sup>	1.03 $\pm$ 0.02

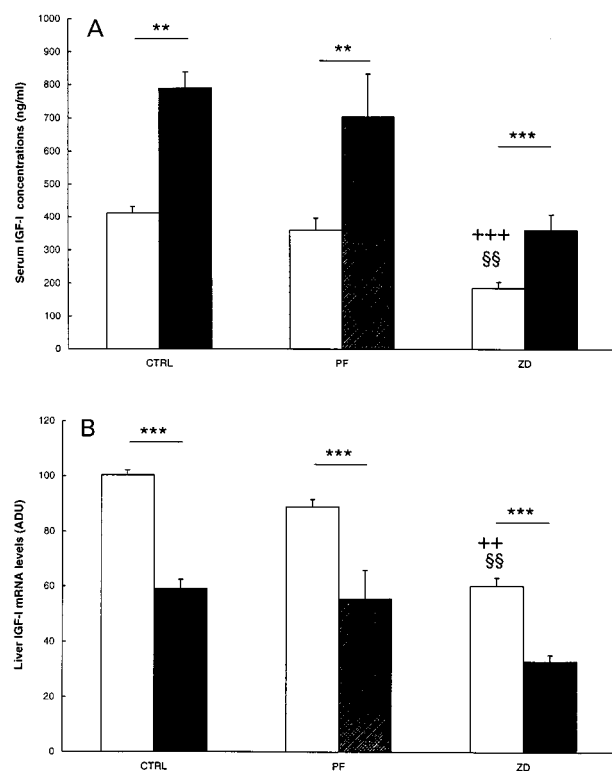
<sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$  and <sup>\*\*\*</sup> $P<0.001$  vs the corresponding untreated group; <sup>+</sup> $P<0.05$ , <sup>++</sup> $P<0.01$  and <sup>+++</sup> $P<0.001$  vs CTRL; <sup>S</sup> $P<0.05$ , <sup>SS</sup> $P<0.01$ , <sup>SSS</sup> $P<0.001$  vs PF.

**Table 3** Effect of a 4-week zinc deficiency and IGF-I infusion during the last experimental week on circulating hormones and glucose. Data are expressed as geometric mean with the 95% confidence interval for GH and insulin and the mean  $\pm$  S.E.M. for glycemia ( $n=6$ /group).

	CTRL	CTRL+IGF-I	PF	PF+IGF-I	ZD	ZD+IGF-I
rGH (ng/ml)	20 (12–38)	4** (1–12)	6+ (2–15)	7 (4–13)	4** (1–13)	1* (1–2)
Insulin (ng/ml)	0.63 (0.35–1.12)	0.69 (0.31–0.55)	0.52 (0.21–1.32)	0.29 (0.14–0.60)	0.26 (0.15–0.46)	0.16* (0.13–0.20)
Glucose (g/l)	1.03 $\pm$ 0.03	1.09 $\pm$ 0.04	0.95 $\pm$ 0.04	1.02 $\pm$ 0.05	1.03 $\pm$ 0.07	0.94 $\pm$ 0.06

\* $P<0.05$ , \*\* $P<0.01$  vs the corresponding untreated group; + $P<0.05$ , ++ $P<0.01$  vs CTRL.

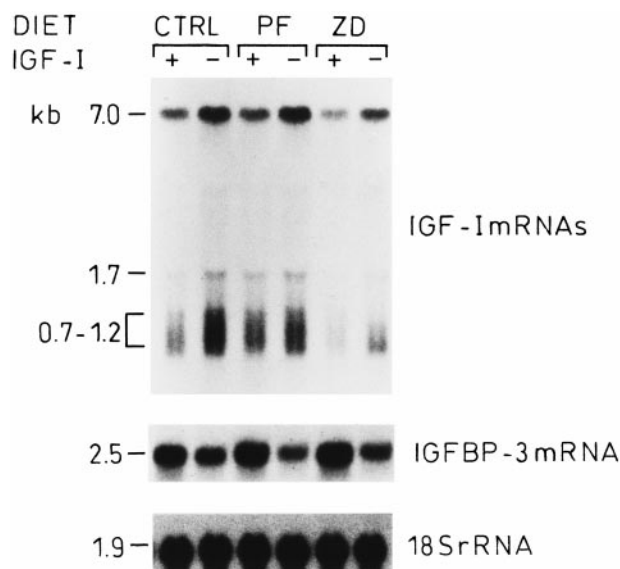
infusion in the three dietary groups (for IGFBP-3: +68% in CTRL,  $P<0.001$ ; +127% in PF,  $P<0.001$ ; +151% in ZD,  $P<0.01$ ; for IGFBP-3 mRNA: +71% in CTRL,  $P<0.05$ ; +94% in PF,  $P<0.01$ ; +121% in ZD,  $P<0.001$ ) (Figs 2, 3 and 4). Serum IGFBP-3 in treated ZD rats was normalized by comparison with untreated CTRL rats (Fig. 3A). There was a good correlation between serum IGF-I and IGFBP-3 ( $r=0.85$ ,  $P<0.0001$ ) in all groups.



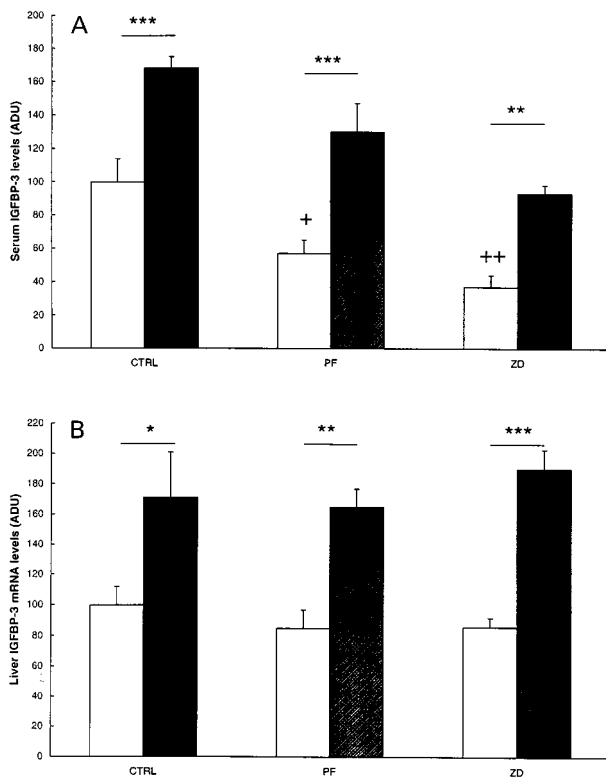
**Figure 1** Serum IGF-I concentrations (A) and liver IGF-I mRNA levels (B) in CTRL, PF and ZD rats, treated (solid bars) or not (open bars) with rhIGF-I (120  $\mu$ g/day). Data are mean  $\pm$  S.E.M. of six rats per group. \*\* $P<0.01$  and \*\*\* $P<0.001$  between the indicated groups; ++ $P<0.01$  and +++ $P<0.001$  vs CTRL; \$\$\$ $P<0.01$  vs PF. ADU=arbitrary scanning densitometric units.

## Discussion

Our results show that the growth retardation caused by dietary zinc deficiency cannot be reversed by IGF-I infusion, despite normalization of circulating IGF-I. This observation strongly suggests that the decrease of serum IGF-I is not the only mechanism responsible for the growth retardation caused by zinc deprivation. When growth retardation results from GH deficiency, as in hypophysectomized rats, IGF-I infusion restores growth, even without normalization of circulating IGF-I (Schoenle *et al.* 1982). Thus, in normally fed rats, IGF-I infusion increases body weight gain and the weight of several organs and therefore mimics the growth-promoting action of GH, in contrast to what is observed in ZD rats. Furthermore our results show that IGF-I may still exert a detectable growth-promoting effect in PF rats which are, however, partially calorie and protein restricted. This

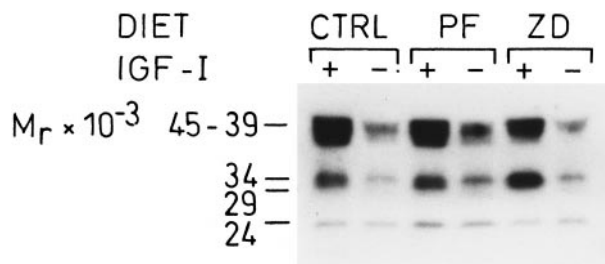


**Figure 2** Northern blots of liver IGF-I mRNA, IGFBP-3 mRNA and 18S rRNA in CTRL, PF and ZD rats, treated or not with rhIGF-I (120  $\mu$ g/day). Representative data are shown for one animal per group.



**Figure 3** Serum IGFBP-3 levels (A) and liver IGFBP-3 mRNA levels (B) in CTRL, PF and ZD rats, treated (solid bars) or not (open bars) with rhIGF-I (120 µg/day). Data are means ± S.E.M. of six rats per group. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  between the indicated groups; + $P < 0.05$  and ++ $P < 0.01$  vs CTRL. ADU=arbitrary scanning densitometric units.

indicates that zinc *per se* is necessary for the full expression of the anabolic action of IGF-I. Because the anabolic response to IGF-I is nevertheless slightly reduced in PF animals by comparison with CTRL, we must recognize that generalized nutritional deprivation may impair the activity of this growth factor. The anabolic action of IGF-I *in vivo* is therefore dependent on the availability not only of protein (Thissen *et al.* 1991a) and calories (Philipps *et al.* 1988), but also of zinc. Since zinc plays a role in many



**Figure 4** Representative ligand blot of serum IGFbps in CTRL, PF and ZD rats, treated or not with rhIGF-I (120 µg/day). Data are shown for one animal per group.

aspects of cell function, there are probably many sites in the IGF-I action that zinc could potentially mediate. The fact that zinc is necessary for fibroblasts to enter into S phase suggests that the stimulation of DNA synthesis by IGF-I may be inhibited in the presence of zinc deficiency (Chesters *et al.* 1989). Such an observation supports the conclusion that zinc is required for the mitogenic action of IGF-I. The growth-promoting effect of IGF-I on spleen is, however, not impaired by the reduced availability of zinc and calories.

Despite the infusion of an identical amount of IGF-I (and even a higher dose per g of body weight), the IGF-I concentrations reached in ZD rats remained lower than those observed in IGF-I treated PF and CTRL rats. This difference in IGF-I concentrations observed may result from accelerated clearance and/or degradation, as described in protein-restricted animals (Thissen *et al.* 1992). This hypothesis is also strengthened by the observed decline in ZD rats of circulating IGFBP-3, the main carrier of IGF-I in the blood. Therefore, we cannot formally exclude that the absence of growth response of ZD rats treated with IGF-I might be related in part to insufficient increase of IGF-I concentrations. Nevertheless, comparable serum levels were sufficient to promote growth in untreated CTRL rats.

Several hypotheses may be proposed to explain the failure of exogenous IGF-I to stimulate growth in ZD animals.

First, the anabolic action of IGF-I might be limited by the action of IGFbps. Although the circulating levels of most major IGFbps are decreased in response to zinc deprivation (Clegg *et al.* 1995, Ninh *et al.* 1997), it is possible that this decline in blood reflects an increase of IGFbps (mainly IGFBP-3) at the cell surface where they could limit the access of IGF-I to the type 1 IGF receptor (Conover 1991). The marked decrease of serum IGFBP-3 levels in response to zinc deprivation together with the minimal changes in liver IGFBP-3 mRNA levels supports this possibility. Alternatively, the decline of IGFBP-3 might result from proteolytic degradation, but this hypothesis is not supported by the recent data reported by Clegg *et al.* (1995). Furthermore, zinc supplementation *in vitro* has been reported to enhance binding of IGF-I to its receptor and decrease the binding to its IGFbps (Mateski & McCusker 1995). It is therefore possible that, in the absence of zinc, IGF-I is preferentially bound to cell surface IGFbps, preventing its interaction with the type 1 IGF receptor.

Secondly, although IGF-I and IGFBP-3 levels are normalized by exogenous IGF-I, the acid-labile subunit (ALS), the third component of the ternary complex, is still probably reduced. Both nutritional restriction (Dai & Baxter 1994, Takenaka *et al.* 1996) and GH deficiency (Labarta *et al.* 1997), which are present in ZD rats, have been reported to decrease ALS production. Furthermore, exogenous IGF-I, by decreasing GH secretion as suggested



by reduced GH plasma levels, might even worsen the decrease of circulating ALS levels (Kupfer *et al.* 1993). Given its crucial role in the formation of the ternary IGF complex, low ALS levels may potentially impair the formation of this complex. As IGF-I bound to the ternary complex seems to represent the most potent anabolic form of circulating IGF-I (Kupfer *et al.* 1993), reduced formation of this complex may contribute to decreased IGF-I bioactivity in ZD rats.

Thirdly, the resistance to IGF-I action in ZD rats may also be mediated by reduced numbers of type 1 IGF receptor. This hypothesis is supported by a recent report showing that decreased DNA synthesis in zinc-depleted 3T3 fibroblasts is associated with decreased IGF-I binding (McDonald & O'Dell 1995). If such a mechanism is operational *in vivo*, it might contribute to the reduced action of IGF-I in ZD rats.

Finally, the possibility remains that zinc impairs cell proliferation and growth by a mechanism independent of IGF-I. Indeed, zinc deprivation might impair cell proliferation at a step distal to IGF-I action, such as reduced thymidine kinase activity or DNA polymerase (Chesters *et al.* 1990). If it is the case, zinc-deficient animals should be resistant to the action of any growth factor.

In conclusion, the decline of circulating IGF-I in zinc-deprived rats is not the only cause of their growth retardation. This seems also, if not predominantly, related to a resistance to the anabolic action of this growth factor.

## Acknowledgements

This work was supported by scholarship from the University of Louvain to N X N and by grants from the Belgian National Fund for Scientific Research (#3-4559.93), from the Fund for Scientific Development, University of Louvain (Belgium) and from the Danone Institute (Brussels, Belgium). J P T is a Research Associate of the National Fund for Scientific Research (Belgium).

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Received 26 February 1998

Revised manuscript received 28 May 1998

Accepted 15 June 1998