Effect of protein restriction on messenger RNA of insulin-like growth factor-I and insulin-like growth factor-binding proteins in liver of ovariectomized rats

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Effects of dietary protein restriction and ovariectomy on plasma concentrations and hepatic messenger RNA (mRNA) of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding proteins (IGFBP) were investigated in young female rats. Ovariectomy increased plasma IGF-I concentration in rats fed on either a 50 g casein/kg diet (protein-restricted diet) or a 200 g casein/kg diet (control diet), but it increased IGF-I mRNA in liver only in the rats fed on the control diet. On the other hand, by Western ligand blot analysis, we observed that ovariectomy increased plasma IGFBP-3 concentration, and decreased plasma IGFBP-4 concentration. Ovariectomy did not affect IGFBP-1 and IGFBP-2 mRNA in liver, but dietary protein restriction significantly increased them, which may correspond to their plasma concentrations. The present results show that ovarian hormones and dietary protein content affect the plasma concentrations of IGF-I, IGFBP-3 and IGFBP-4 and hepatic mRNA of IGF-I, IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 in different manners.

Insulin-like growth factor-I: Insulin-like growth factor-binding proteins: Protein restriction: Ovariectomy

In a preceding paper (Higashi *et al.* 1996) we showed that femoral messenger RNA (mRNA) of bone matrix proteins and growth factors, which play important roles in bone formation, are affected by both dietary protein content and ovariectomy. In particular, the mRNA of insulin-like growth factor (IGF)-I, IGF-II and their binding proteins reacted to ovariectomy and protein restriction.

The quantity and the nutritional quality of dietary proteins are well known to affect plasma IGF-I concentration greatly (Prewitt *et al.* 1982; Isley *et al.* 1983; Takahashi *et al.* 1990). Concomitantly IGF-I mRNA in liver is affected greatly as well (Moats-Staats *et al.* 1989; Thissen *et al.* 1991*a*; Miura *et al.* 1992). We and others have suggested that IGF-I regulates body protein metabolism in response to dietary proteins. On the other hand, IGF-I and IGF-II have been shown to circulate in blood complexed with specific proteins referred to as IGF-binding proteins (IGFBP) (Cohen & Nissley, 1975; Hintz & Liu,

1977). Six distinct IGFBP from man and the rat have already been purified and their complementary DNA (cDNA) have been cloned (Martin & Baxter, 1986; Brinkman *et al.* 1988; Brown *et al.* 1989; Mohan *et al.* 1989; Shimasaki *et al.* 1990, 1991*a,b*). These IGFBP, in purified or recombinant form, greatly modulate the biological activity of IGF-I or IGF-II. We and others (Orlowski *et al.* 1990; Murphy *et al.* 1991; Thissen *et al.* 1991*b*; Umezawa *et al.* 1991) have reported that plasma concentrations of IGFBP are also affected significantly by dietary factors. Therefore, the effect of IGF-I on the target tissues must be regulated in a complex manner through the changes in the concentration of IGF-I itself and changes in the concentrations of IGFBP.

The importance of local synthesis of IGF and IGFBP for cell proliferation and cell differentiation has repeatedly been stressed by many investigators (Murphy, 1991; Baylink *et al.* 1993; Hammond *et al.* 1993; Nestler, 1993;

Vinik *et al.* 1993). In the preceding paper, we showed the effect of dietary protein restriction and ovariectomy on the mRNA of IGF and IGFBP in femur, and mentioned the biological significance of IGF and IGFBP which are produced locally. However, as IGF-I and some IGFBP are primarily produced by liver and secreted into the bloodstream, it is possible that these proteins can regulate bone metabolism. The possible endocrine role of IGF and IGFBP must be determined. In the present paper, we report the changes in the plasma concentrations of IGF and IGFBP and their mRNA levels in liver under protein restriction and ovariectomy.

Materials and methods

Materials

Human recombinant IGF-I and anti-human IGF-I antibody were kindly donated by Dr M. Niwa, Fujisawa Pharmaceutical Co. (Osaka, Japan). Normal rabbit serum and anti-rabbit γ-globulin goat antibody were obtained from Daiichi Radioisotope Laboratory (Tokyo, Japan). ^{125}I-labelled IGF-I was prepared from Na^{125}I and IGF-I according to a mild chloramine T method as modified by Umezawa *et al.* (1991). The cDNA of rat IGF-I, IGFBP-1 and IGFBP-3 were prepared as we reported previously (Kato *et al.* 1990; Takenaka *et al.* 1991, 1993). Rat IGFBP-2 cDNA and IGFBP-4 cDNA were kindly given by Dr A.J. D'Ercole, The University of North Carolina (NC, USA). Other chemicals were of the reagent grade available commercially.

Animals

Animals were the same as those in the preceding paper (Higashi *et al.* 1996). Briefly, 7-week-old female Wistar-strain rats were obtained from Charles River Japan (Kanagawa, Japan) and fed on a control diet containing 200 g casein/kg diet (200C diet, Higashi *et al.* 1996) until the operation. The rats were given the diet from 09.00 to 17.00 hours and water *ad libitum*. After 4 d, the rats were either ovariectomized bilaterally or sham-operated, and then were divided into two dietary groups; (A) those fed on the control diet (initial body weight: sham-operated rats, 193 (SE 2) g, *n* 5; ovariectomized rats, 189 (SE 2) g, *n* 5) and (B) those fed on a 50 g casein/kg diet (50C diet, Higashi *et al.* 1996; protein-restricted diet) (initial body weight: sham-operated rats, 186 (SE 5) g, *n* 5; ovariectomized rats, 190 (SE 5) g, *n* 5). The food intake of the control group was paired with that of the protein-restricted group of the same operation, i.e. the food intake of the ovariectomized or sham-operated rats fed on the 50C diet was measured individually and the same amount of the 200C diet was fed to the paired rats on the next day. At 3 weeks later the rats were fed at 10.00 hours and killed at 11.30 hours by decapitation under pentobarbital anaesthesia (50 mg pentobarbital/kg body weight). Arterial blood was collected in Na$_2$EDTA and plasma was prepared as described previously (Takahashi *et al.* 1990). The plasma was kept at $-20°$ until analysis. The liver was quickly excised, frozen in liquid N$_2$ and kept at $-80°$ until RNA preparation.

IGF-I radioimmunoassay

The total IGF-I concentration was determined after treatment of the plasma with an acid–ethanol mixture as described previously (Takahashi *et al.* 1990), because, in these experiments, plasma concentration of total IGF-I measured after acid–ethanol treatment was not significantly different from total IGF-I concentration measured after acid–gel permeation chromatography according to the method of Crawford *et al.* (1992). Radioimmunoassay for IGF-I was performed as follows. Samples (0·1 ml) after acid–ethanol treatment were mixed with 0·1 ml IGF-I antibody solution and 0·5 ml buffer solution for radioimmunoassay (Dulbecco's phosphate-buffered saline solution consisting of (g/l): NaCl 8, KCl 0·2, Na$_2$HPO$_4$ 1·15 and NaH$_2$PO$_4$ 0·2, supplemented with 25 mM-EDTA and 50 g bovine serum albumin (BSA)/l). This solution was kept at $4°$ for 24 h, then 0·1ml each of rabbit serum (fifty times diluted) and anti-rabbit γ-globulin goat antibody (ten times diluted) were added to the solution. This mixture was allowed to stand at $4°$ for 2–3 h and then centrifuged at 2000 g for 30 min at $4°$. The radioactivity in the precipitate was counted with a γ-counter (Aloka Auto Well Gamma System ARC-500, Aloka, Tokyo, Japan). Recombinant human IGF-I was used as the standard for IGF-I determination.

Total RNA preparation

The total RNA was prepared from liver according to the method described previously (Miura *et al.* 1992).

Northern hybridization analysis

The methods used to label probes and perform Northern hybridization were as described previously and in the preceding paper (Miura *et al.* 1992; Higashi *et al.* 1996).

Western ligand blot analysis of IGFBP in plasma

Plasma concentrations of IGFBP were determined by Western ligand blot analysis by the method of Umezawa *et al.* (1991). At the time of analysis the plasma was mixed with an equal volume of 2 × concentrated Laemmli's sample buffer solution (the original 1 × Laemmli's solution contained 50 g SDS/l, 100 ml glycerol/l, 0·06 M-Tris-HCl, pH 6·8 and 0·2 ml bromophenol blue/l) and heated at $65°$ for 15 min. This sample was electrophoresed on 125 g/l polyacrylamide gel containing 1 g SDS/l without reducing agents. After electrophoresis, proteins were transblotted to a nitrocellulose filter in the transfer buffer solution (2·9 g/l Tris, 14·5 g/l glycine in 200 ml/l methanol, pH 8·3) at 200 mA for 4·5 h using the transblot system (Bio-Rad, CA, USA). The filter was soaked in 30 ml/l NP-40 saline solution (saline solution consists of 0·15 M-NaCl and 0·01 M-Tris–HCl, pH 7·4) for 30 min and further blocked in 30 g/l BSA in saline solution at $4°$ for 12 h. The filter was washed by shaking in 1 ml/l Tween 20 in saline at $4°$ for 10 min. Then the filter was put in 1 ml/l Tween 20, 40 g/l BSA with [^{125}I]IGF-I (4000 cpm) in saline at $4°$ for 10 h.

Table 1. Body-weight gain, food intake and plasma concentrations of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBP) in ovariectomized and sham-operated rats fed on diets containing 50 g (50C) or 200 g casein/kg (200C)

(Mean values for five rats with their pooled standard error)

Diet...	50C		200C			Statistical significance of effect of:		
Treatment...	Sham-operated	Ovariectomized	Sham-operated	Ovariectomized	SEM	Diet (D)	Ovariectomy (O)	O × D
Body-wt gain (g)	40†	60†‡‡	49	75‡‡	5	*	**	NS
Food intake (g/d)	14·9	17·1‡‡	14·9	17·1‡‡	0·6	NS	**	NS
Total IGF-I (ng/ml)	8608	10573‡	9820	11256‡	629	NS	*	NS
IGFBP-3§	90·3	106·9‡	100·0	135·5‡	11·1	NS	*	NS
IGFBP-4§	95·0	62·3‡	100·0	74·6‡	10·8	NS	*	NS

$*P < 0.05$, $**P < 0.01$ by two-way ANOVA.
Mean values were significantly different from those for the 200C group: † $P < 0.05$ (Tukey's method).
Mean values were significantly different from those for the sham-operated group: ‡ $P < 0.05$, ‡‡ $P < 0.01$ (Tukey's method).
§ The IGFBP levels were determined by quantification of the Western ligand blotting shown in Fig. 2 by a Fujix Bas 2000 system (Fuji Film Co., Kanagawa, Japan). Results are expressed as the relative radioactivity of the bands, taking the mean of the sham-operated 200C group as 100.

After the filter was washed twice by shaking in 1 ml/l Tween 20 in saline at 4° for 10 min and twice in saline, it was air-dried and autoradiographed. The radioactivity of the filter was also determined by Fujix Bas 2000 Bio Image Analyzer (Fuji Film Co., Tokyo, Japan), and the results were expressed as the relative radioactivity of the bands taking the mean of the sham-operated 200C group as 100.

Statistical analysis

The results were analysed statistically by two-way ANOVA (Snedecor & Cochran, 1967). When the difference among the groups was significant, Tukey's least significant difference method was employed to show the difference among the mean values.

Results

The effects of protein restriction and ovariectomy on plasma IGF-I concentration and on the gene expression of IGF-I in liver

Table 1 shows the plasma IGF-I concentration in the protein-restricted and ovariectomized rats. Ovariectomy significantly increased the plasma concentration of total IGF-I after acid–ethanol extraction; on the other hand, protein restriction did not affect it. The total IGF-I concentration was correlated with the changes in body-weight gain (r 0·457, $P < 0·05$).

Fig. 1 and Table 2 show the changes in IGF-I mRNA content in liver of the ovariectomized and sham-operated rats fed on the two diets. The effects of ovariectomy and protein restriction interacted significantly, because ovariectomy increased IGF-I mRNA in liver in the 200C diet groups, but not in the 50C diet groups. This tendency was observed equally for mRNA for all four species of IGF-I (Fig. 1).

The effects of protein restriction and ovariectomy on plasma IGFBP concentration and on the gene expression of IGFBP in liver

Fig. 2 and Table 1 show the effect of ovariectomy and/or protein restriction on the plasma concentrations of IGFBP

in the rats determined by Western ligand blot analysis. Ovariectomy showed contrasting effects on IGFBP-3 and IGFBP-4, as plasma IGFBP-3 concentration was increased, but IGFBP-4 concentration was decreased by ovariectomy. On the other hand, protein restriction had no effect on these concentrations (Fig. 2 and Table 1).

Fig. 3 and Table 2 show the changes in IGFBP mRNA in liver. Ovariectomy caused variable effects on IGFBP-1 mRNA content, increasing the IGFBP-1 mRNA in three of five rats fed on the 200C diet in this study. Dietary protein restriction increased IGFBP-1 mRNA content when the rats were sham-operated, but not when they were ovariectomized (Table 2). Ovariectomy did not show any significant effect on IGFBP-2 and IGFBP-3 mRNA in liver; on the other hand, protein restriction significantly increased IGFBP-2 mRNA, and decreased IGFBP-3 mRNA content, and this effect interacted with that of ovariectomy, resulting in decreased mRNA content only in ovariectomized rats fed on the 200C diet.

Discussion

In the present studies we showed that ovariectomy and dietary protein restriction affected plasma concentrations of IGF-I and some IGFBP, and their corresponding mRNA in liver in different ways.

Ovariectomy increased the plasma IGF-I concentration and this was correlated with the body-weight changes as well as IGF-I mRNA contents in liver (Tables 1 and 2). These results suggest that ovarian hormone deficiency leads to animal growth through increasing hepatic IGF-I synthesis. However, we observed increased food consumption in ovariectomized rats in this experiment. Since not only the quality, but also the quantity of protein is known to influence the plasma IGF-I concentration (Takahashi *et al.* 1990; for review, see Thissen *et al.* 1994), we could not rule out the possibility that these changes in plasma IGF-I concentration and hepatic IGF-I mRNA contents were explained by food consumption. Aerssens *et al.* (1993) and Sato *et al.* (1993) also reported that ovariectomy increased the plasma IGF-I concentration. In the experiment of Sato *et al.* (1993) the food consumption of ovariectomized rats was matched with that of sham-operated rats. Thus, the
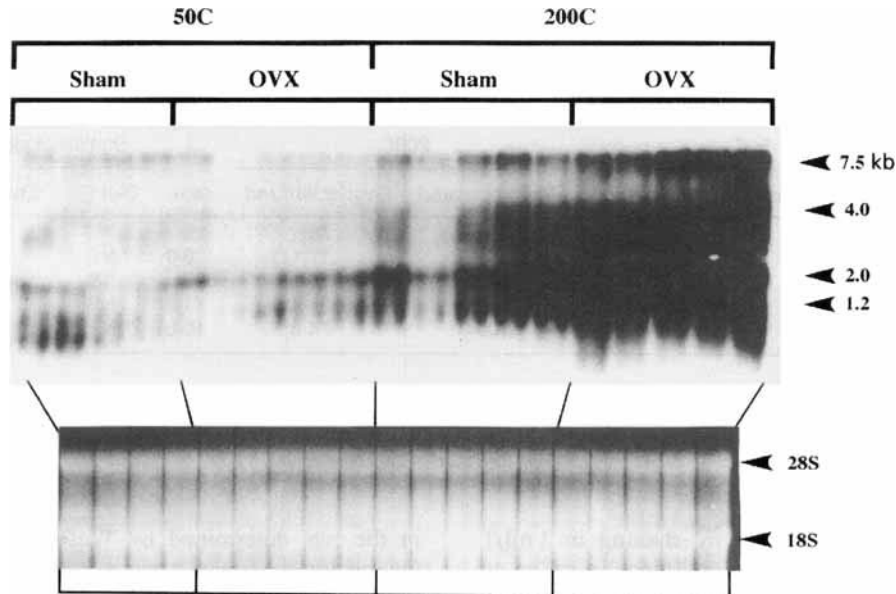
Fig. 1. Northern blot analysis of insulin-like growth factor-I messenger RNA in liver from sham-operated (Sham) and ovariectomized (OVX) rats fed on diets containing 50 (50C) or 200 g casein/kg (200C) for 7 d. For details of procedures, see pp. 448–449. A 30 µg portion of total RNA from one rat was applied to each lane. The positions of molecular markers are indicated on the right. Bands of ribosomal RNA stained by ethidium bromide (bottom, sizes shown on the right) demonstrate equal amounts of RNA per lane.

increased plasma IGF-I concentration in the ovariecto-mized rats could not have been the result of increased food consumption, but must have been related to oestrogen or ovarian hormone deficiency. From these results, we assume that ovarian hormone deficiency directly induces hepatic gene expression of IGF-I, thereby inducing an increase in IGF-I production in liver.

Furthermore, we found that the effect of protein restriction interacted with that of ovariectomy on the IGF-I mRNA content in liver. Dietary protein restriction decreased the mRNA content as previously reported (Thissen *et al.* 1994), and ovariectomy increased the mRNA content of the 200C diet groups, but not that of 50C diet groups. These results suggest that oestrogen suppresses gene expression of IGF-I in liver, but, when the dietary protein is restricted, this suppressive effect of oestrogen is completely abolished. In liver, Murphy & Friesen (1988) and Krattenmacher *et al.* (1994) reported that chronic oestrogen treatment reduced the IGF-I mRNA content. They concluded that the oestrogen effect was indirect and might be caused by oestrogen depression of growth hormone-dependent IGF-I production in liver. These results are comparable to our observation that chronic oestrogen deficiency increased the hepatic IGF-I mRNA content. We observed that ovariectomy did not influence the IGF-I mRNA when the protein intake was restricted. This observation, together with the finding that
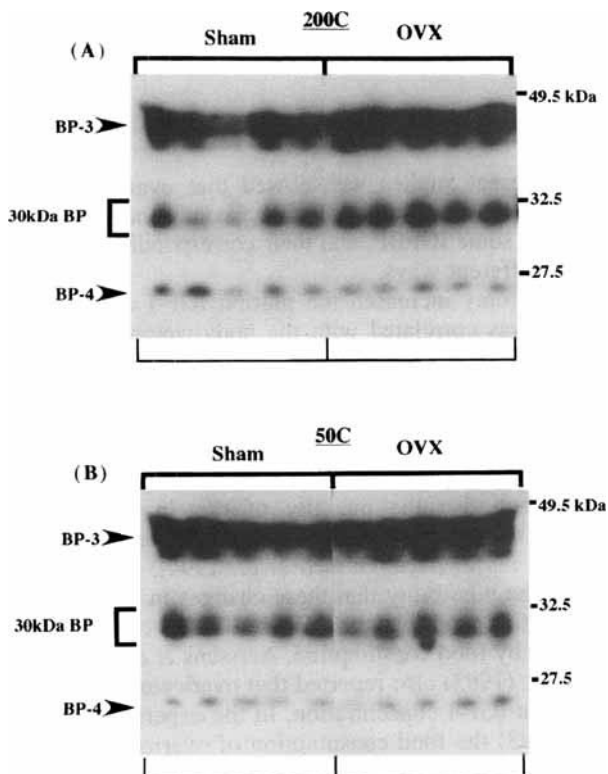
Fig. 2. Western ligand blot analysis of insulin-like growth factor-binding proteins in plasma from sham-operated (Sham) and ovariectomized (OVX) rats fed on diets containing (A) 50 g casein/kg (50C) and (B) 200 g casein/kg (200C). Plasma (5 µl) was subjected to SDS-PAGE and after electrophoresis proteins were transferred to a nitrocellulose filter. The proteins which bound [^{125}I] insulin-like growth factor-I were detected by a Fujix Bas 2000 system (Fuji Film Co., Kanagawa, Japan). Each lane corresponds to the plasma from one rat.

Table 2. IGF-binding protein (IGFBP) messenger RNA (mRNA) contents‡ in the livers of ovariectomized and sham-operated rats fed on diets containing 50 (50C) or 200 g casein/kg (200C)

(Mean values for five rats with their pooled standard error)

Diet...	50C		200C			Statistical significance of effect of:		
Treatment...	Sham-operated	Ovariectomized	Sham-operated	Ovariectomized	SEM	Diet (D)	Ovariectomy (O)	O × D
IGFBP-1 mRNA	1499·9^a	988·0^{ab}	100·0^c	540·9^{bc}	165·9	**	NS	*
IGFBP-2 mRNA	303·2†	450·9†	100·0	180·6	91·7	*	NS	NS
IGFBP-3 mRNA	60·8†	74·6†	100·0	124·6	10·8	**	NS	NS
IGFBP-4 mRNA	86·9^{AB}	94·7^A	100·0^A	58·0^B	5·7	NS	*	**

^{a,b,c,A,B,C} Mean values within a row not sharing a common superscript letter were significantly different: $^{a,b,c}P < 0.05$, $^{A,B,C}P < 0.01$ (Tukey's method).
*$P < 0.05$, **$P < 0.01$ by two-way ANOVA. Mean values were significantly different from those for the 200C group. †$P < 0.05$, ††$P < 0.01$.
‡The mRNA levels were determined by quantification of the Northern blot analyses shown in Figs. 1 and 3 using a Fujix Bas 2000 system (Fuji Film Co., Kanagawa, Japan). Results are expressed as relative radioactivity of the bands, taking the mean of the sham-operated 200C group as 100.
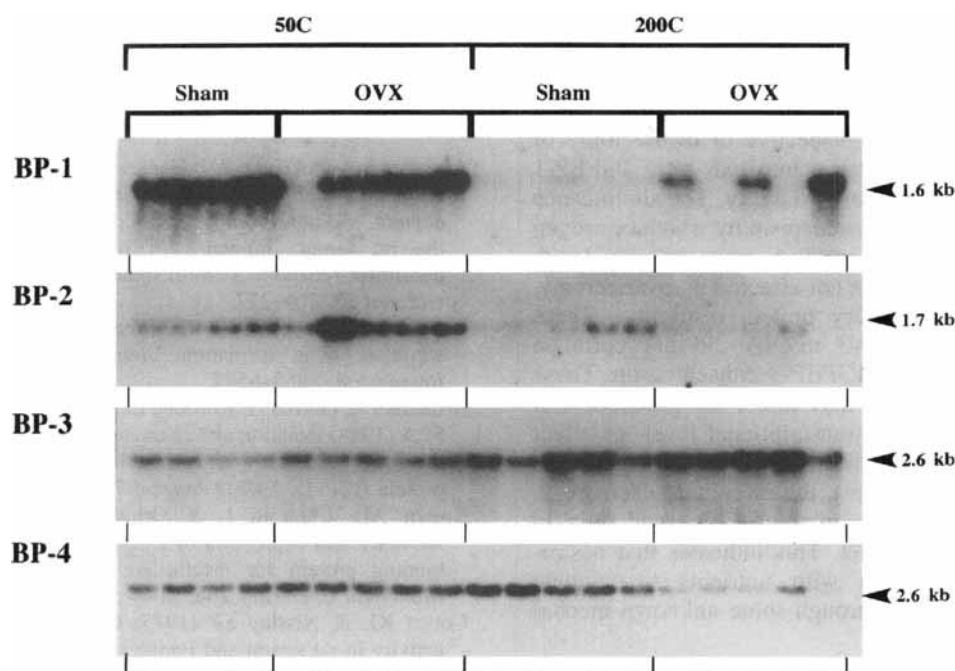

Fig. 3. Northern blot analysis of insulin-like growth factor-binding protein (BP) messenger RNA in liver from sham-operated (Sham) and ovariectomized (OVX) rats fed on diets containing 50 (50C) or 200 g casein/kg (200C) for 7 d. For details of procedures, see pp. 448–449. A 30 µg portion of total RNA from one rat was applied to each lane.

protein malnutrition impaired growth hormone dependent IGF-I production by a post-growth hormone receptor event (Maes et al. 1988), raises the possibility that a decrease in IGF-I gene expression, induced either by high levels of oestrogen or low dietary protein, is a growth hormone-mediated occurrence.

Despite these observations about the effect of protein restriction on IGF-I mRNA, the plasma IGF-I concentration was not significantly affected by protein restriction. We and others have already shown that protein malnutrition causes a decrease in liver IGF-I mRNA (Moats-Staats et al. 1989; Thissen et al. 1991a; Muira et al. 1992). The finding that plasma IGF-I concentration did not correlate with the hepatic IGF-I mRNA may be due to a change in translation of the mRNA, or to altered IGFBP profile.

The biological activity and the half-life of IGF is believed to be modulated by IGFBP. In some cases, ovariectomy and dietary protein restriction changed the plasma concentrations of IGFBP which were determined by Western ligand blotting (Fig. 2 and Table 1). Ovariectomy increased the plasma IGFBP-3 concentration. The bands at about 30 kDa in Western ligand blotting consisted of at least five IGFBP including IGFBP-1, IGFBP-2, glycosylated IGFBP-4, IGFBP-5 and IGFBP-6. Therefore, it is impossible to say which IGFBP at about 30 kDa was affected by protein restriction and ovariectomy. Non-glycosylated IGFBP-4 at 20 kDa was clearly separated from the IGFBP at about 30 kDa and ovariectomy decreased the plasma concentration of non-glycosylated IGFBP-4. Kalu et al. (1994) showed that ovariectomy

increased, low-dose 17β-oestradiol restored, and high-dose 17β-oestradiol reduced serum IGFBP-3 concentrations compared with sham-operated rats, and these treatments did not affect serum levels of other small IGFBP. Our results for IGFBP-3 are well in accordance with their reports. From the results for plasma IGFBP-3 and IGF-I (see p. 449), we conclude that plasma IGFBP-3 concentration is well correlated with plasma IGF-I level through some mechanism, such as formation of the complex of IGF-I, IGFBP-3 and the acid labile subunit, possibly to prolong the half-life of both IGF-I and IGFBP-3 in plasma. Furthermore we showed that ovarian hormone deficiency could influence plasma IGFBP-4 concentration. These results suggest that plasma IGF-I which is bound to IGFBP-3 increased and that bound to IGFBP-4 decreased under oestrogen deficiency.

We also measured the mRNA content of each IGFBP in liver to examine the effects of ovariectomy and/or dietary protein restriction. The levels of liver IGFBP-1 and IGFBP-2 mRNA increased significantly under dietary protein restriction, irrespective of ovariectomy or sham-operation. Among ovariectomized rats, IGFBP-1 mRNA contents showed great variability. The significance of this observation, and the mechanism by which oestrogen regulates IGFBP-1 gene expression, must be elucidated. IGFBP-3 mRNA content was not affected by ovariectomy, but was decreased by dietary protein restriction. These changes in hepatic IGFBP-3 mRNA did not correlate with the change in plasma IGFBP-3 concentration. These results show that oestrogen may affect the production of IGFBP-3 in liver at a post-transcriptional level, or affect its production in organs other than liver, or change its stability in plasma. In contrast, the prominent decrease in IGFBP-4 mRNA content in liver was observed only in the rats fed on the 200C diet. This indicates that oestrogen works in cooperation with nutrients to regulate IGFBP-4 gene expression through some unknown mechanisms.

IGF-I and IGFBP are locally produced in various tissues and organs, and the importance of their local action has been stressed by many investigators (Jones & Clemmons, 1995). For example, in the uterus, oestrogen-dependent production of IGF-I is known to be important to maintain functions of this organ (Murphy, 1991). In bone, we reported that ovarian hormone deficiency and dietary proteins affected IGF-I and IGFBP gene expression, and that they could regulate bone metabolism (Higashi *et al.* 1996). On the other hand, endocrine IGF-I and IGFBP are also shown to regulate metabolism in various tissues and organs. For example, plasma IGF-I concentration correlates well with the rate of whole-body protein synthesis (Nam *et al.* 1990). From these results and those from previous studies showing that ovariectomy induced an increase in bone turnover, we assume that increasing formation of IGF-I and IGFBP-3 complex as well as decreasing IGFBP-4 in plasma, caused an increase in IGF-I bioactivity under oestrogen or ovarian hormone deficiency. On the other hand, IGF-I bioactivity is supposed to be suppressed by decreasing formation of IGF-I and IGFBP-3 complex as well as increasing plasma IGFBP-1 and IGFBP-2 under protein malnutrition.

In the present study we investigated the effects of ovariectomy and dietary protein restriction on the plasma concentrations of IGF-I and IGFBP, and the mRNA contents of IGF-I and IGFBP in liver. From these observations we suggest that plasma IGF and IGFBP, whose production is affected by oestrogens or ovarian hormones and dietary proteins, play very important roles in the regulation of animal growth and metabolism.

Acknowledgements

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