Insulin-like growth factor binding proteins in the rat uterus and their regulation by oestradiol and growth hormone

C. Yallampalli¹, S. Rajaraman² and M. Nagamani¹

¹Department of Obstetrics and Gynecology and ²Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77550, USA

The rat uterus has previously been shown to be a site of insulin-like growth factor I (IGF-I) production and reception. The purpose of this study was to explore the possibility that the rat uterus can also hormonally regulate elaboration of IGF-binding proteins (IGFBPs). Uteri from adult ovariectomized rats were perfused, rinsed thoroughly and extracted. Western ligand blotting of SDS-polyacrylamide-fractionated uterine extracts revealed several bands of IGFBPs with molecular masses of 24, 28, 30–32 and 38–42 kDa; the 28 kDa protein was not detected in the serum. Hypophysectomy caused a marked decrease in 38–42 and 30–32 kDa proteins which was reversed by systematic treatment with growth hormone ($2 \times 120 \mu\text{g}$ per rat per day for 3 days). The 28 and 24 kDa proteins, however, were not altered by growth hormone. Oestradiol ($1 \mu\text{g}$ per rat per day for 3 days) induced more than a 50% decrease in both 38–42 and 28 kDa proteins, irrespective of the growth hormone status in ovariectomized rats. These studies disclose the multiplicity of uterine IGFBPs and show the ability of growth hormone and, more importantly, oestradiol to regulate these proteins. The ability of oestradiol to attenuate the IGFBPs in the uterus may enhance the access of endogenously produced IGFs to its cognate cell receptors and hence its cellular hormone action.

Introduction

Insulin-like growth factors (IGFs) are small molecular mass peptides that stimulate replication of cells in many tissues (Van Wyk, 1985). In all the extracellular and interstitial fluids analysed so far, the IGFs are complexed to high-affinity binding proteins (Hardouin *et al.*, 1987). These IGF-binding proteins (IGFBPs) are members of a family of proteins of various sizes that are soluble, secreted by a variety of tissues and bind both IGF-I and IGF-II but not insulin (Binoux *et al.*, 1986). Although the role(s) of tissue-derived IGFBPs is unclear, both inhibition (Ritvos *et al.*, 1988) and stimulation (Clemmons *et al.*, 1986) of IGF binding and action have been reported. The molecular masses for the rat IGFBPs on SDS polyacrylamide gels are: IGFBP-1, 28 kDa; IGFBP-2, 30–32 kDa and IGFBP-3, 38–45 kDa and the sequences of these three classes of rat IGFBP have been reported (Brown *et al.*, 1989; Shimasaki *et al.*, 1989; Murphy *et al.*, 1990). The sequences of three more classes of rat IGFBP, IGFBP-4, -5, and -6 have been published (Shimasaki *et al.*, 1990; Ling *et al.*, 1991; Shimasaki *et al.*, 1991) with predicted molecular masses of 24, 28–29 and 21–22 kDa, respectively.

Although the relevance of IGFBPs to uterine physiology remains unclear, one of the IGFBPs, IGFBP-1, is produced by the nonpregnant human endometrium during the late secretory phase (Rutanen *et al.*, 1986; Julkunen *et al.*, 1989) and by decidual cells of the pregnant uterus (Rutanen *et al.*, 1985; Julkunen *et al.*, 1989). Expression of mRNA encoding IGFBP-2 and -3 in human endometrium (Giudice *et al.*, 1991) and secretion of multiple

forms of IGFBPs by decidual cells (Clemmons *et al.*, 1990) have been reported. In this article, we report the presence of various IGFBPs in rat uterine extracts and their modulation by growth hormone and oestradiol.

Materials and Methods

Materials

¹²⁵I-labelled IGF-I was purchased from Amersham Corporation (Arlington Heights, IL); ovine growth hormone (NIADDK-oGH-15) was provided by National Hormone and Pituitary Program of the National Institutes of Health (Bethesda, MD). Oestradiol, sesame oil, radioimmunoassay grade BSA and other analytical grade chemicals were purchased from Sigma Chemical Co (St Louis, MO). Ketamine (Ketalar) was purchased from Parke-Davis (Morris Plains, NJ). Recombinant IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA).

Animals and treatments

Female Sprague-Dawley rats, hypophysectomized at 200–225 g body weight, were obtained from Harlan-Sprague-Dawley Co, Houston, TX. Pituitary-intact female rats of the same strain were also obtained from Harlan-Sprague-Dawley at 150–160 g body weight. All the animals were housed in temperature (22°C) and light controlled conditions, with free access to food and water. The hypophysectomized rats were also given 2% glucose in addition to water.

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Fig. 1. Western ligand blotting of (a) serum and (b) uterine extracts from adult ovariectomized rats. Aliquots of serum (5 μ l) or uterine extracts (160 μ g protein) were fractionated by SDS-PAGE, transferred to nitrocellulose and labelled with ^{125} I-labelled insulin-like growth factor I (IGF-I). The nitrocellulose was exposed to X-ray film for 5 days. Each lane is from a representative animal in the pituitary-intact (lanes 1 and 2), hypophysectomized (lanes 3 and 4), or hypophysectomized plus growth hormone (lanes 5 and 6) group that received either saline (lanes 1, 3 and 5) or 1 μ g oestradiol (lanes 2, 4 and 6). The positions of 22, 31 and 42.7 kDa marker proteins are shown.

All the rats were ovariectomized under ketamine (75 mg kg⁻¹ body weight) anaesthesia and allowed at least 7 days to recover from the surgery. At the start of injections to the animals, hypophysectomized-ovariectomized rats had been hypophysectomized for 10–14 days. Hypophysectomized-ovariectomized rats received twice daily s.c. injection of ovine growth hormone (120 μ g, in 0.03 mol NaHCO₃ l⁻¹ with 0.15 mol NaCl l⁻¹) or vehicle only. The pituitary-intact ovariectomized rats received vehicle only. All these animals also received daily s.c. injection of 0.2 ml of sesame oil with or without 1 μ g oestradiol. After 3 days of treatment, under ketamine anaesthesia, blood samples were collected and whole animal perfusions were carried out using heparinized saline to remove blood from the uterus. Saline was infused through a catheter placed in the jugular vein using a peristaltic pump and the perfusate was released through a puncture in the posterior vena cava.

The animals were killed and the uteri were cleaned, slit open longitudinally and rinsed thoroughly in cold Tris-HCl (10 mmol l⁻¹ pH 7.4), 10 mmol MgCl₂ l⁻¹, 1 mmol EDTA l⁻¹ and 0.2 mmol phenylmethyl sulfonylfluoride l⁻¹. Uteri from individual rats were snap frozen in liquid nitrogen and stored at -80°C until used. The uteri from individual rats were homogenized in Tris-HCl buffer at a tissue buffer ratio of 1 g: 4 ml with three 15 s bursts, at a setting of 60 in a Tekmar tissuemizer (Tekmar Co, Cincinnati, OH) with cooling intervals of 1 min. The homogenates were centrifuged at 106 000 g for 90 min and aliquots of supernatant extracts were removed for protein and haemoglobin estimation.

Insulin-like growth factor binding proteins in the uterine extracts and serum were analysed by western ligand blotting (Hossenlopp *et al.*, 1986). Briefly, the samples were treated to 100°C for 5 min in 4 $\times$ Laemmli sample buffer under nonreducing conditions and loaded onto 12% SDS polyacrylamide gels. Gel electrophoresis was performed with Protean II apparatus (Bio Rad, Richmond, CA) at 35 mA per gel until the dye front reached the bottom of the gel. After electrophoresis, gels washed in Towbin buffer (0.025 mol Tris-base l⁻¹, 0.192 mol glycine l⁻¹, 20% methanol) to remove SDS and proteins electroblotted onto nitrocellulose membranes (0.5 μ m) using Towbin

buffer in a Trans-blot apparatus (BioRad) at constant 30 V for 16 h at 4°C (Hossenlopp *et al.*, 1986). Membranes were washed at 4°C on a rocking-platform with (1) TS buffer (150 mmol NaCl l⁻¹ and 10 mmol Tris-HCl l⁻¹, pH 7.4, (containing 0.5 mg NaN₃ ml⁻¹, 3% NP40) for 30 min; (2) TS buffer plus 1% BSA for 2 h; and (3) TS buffer plus 0.1% Tween-20 for 20 min. The membranes were incubated in a heat-sealable plastic bag with ^{125} I-labelled IGF-I (1.5 $\times$ 10⁶ c.p.m.) in TS buffer containing 1% BSA and 0.1% Tween-20 for 16–24 h at 4°C. After incubation, membranes were washed twice with TS buffer with 0.1% Tween-20 (15 min each) and then twice with TS buffer alone, air dried and autoradiographically developed. The autoradiograms were scanned using a laser densitometer (Pharmacia LKB Instruments, Alameda, CA) and absorbance units $\times$ millimetre (AV $\times$ mm) were measured for each of the major IGF-BPs. Molecular mass estimates were based on prestained protein standards (Biorad). The amount of blood contamination in the uterine extracts as determined by haemoglobin assay (Crosby and Furth, 1956) was less than 0.001%.

Statistical analysis

Data were analysed using analysis of variance and the difference between means was tested using Duncan's multiple range test.

Results

IGFBPs in the uterine extracts

Ligand blot analysis of uterine extracts was performed to determine the forms of IGF-BP present in the rat uterus. Autoradiographs of the nitrocellulose membranes revealed both serum and uterine extracts that contained several bands of IGF-BPs. These binding proteins were specific for IGF-I, as binding was totally displaced by radioinert IGF-I (100 μ g ml⁻¹) (data not shown). Autoradiographs of normal rat serum revealed three major groups of IGF-BPs. The most abundant group contained several bands with molecular masses ranging from 38 to 42 kDa (Fig. 1). Since these bands were not resolved

Table 1. Effects of growth hormone and oestradiol on different forms of insulin-like growth factor binding proteins (IGFBPs) in uterine extracts

		IGFBP (scanning units) (kDa)			
Treatment group	n	38–42	30–32	28	24
Pituitary intact–ovariectomized					
– Oestradiol	4	314 ± 68 ^a	413 ± 43 ^b	283 ± 23 ^a	306 ± 31 ^a
+ Oestradiol	4	120 ± 31 [*]	381 ± 24	141 ± 15 [*]	271 ± 40
Hypophysectomized–ovariectomized					
– Oestradiol	4	106 ± 15 ^b	56 ± 8 ^b	263 ± 26 ^a	301 ± 41 ^a
+ Oestradiol	4	53 ± 11 [*]	117 ± 15 [*]	91 ± 12 [*]	229 ± 38
Hypophysectomized–ovariectomized + growth hormone					
– Oestradiol	4	194 ± 23 ^c	110 ± 18 ^c	254 ± 17 ^a	274 ± 4 ^a
+ Oestradiol	4	92 ± 12 [*]	121 ± 10	62 ± 8 [*]	248 ± 38

Values are scanning densitometric units of autoradiographs of Western ligand blots (with ¹²⁵I-labelled IGF-I) presented as means ± SEM for four animals in each group. Samples from experimental groups were run on gels on the same day and exposed together on the same radiograph.

*Significantly different ($P < 0.05$) from animals not receiving oestradiol within pituitary intact, hypophysectomized or hypophysectomized + growth hormone group. Values with different superscripts within each column differ significantly ($P < 0.05$).

completely, they were grouped for scanning densitometric analysis. The second group of IGFBPs consisted of proteins with molecular masses of 30–32 kDa. The third group consisted of a single band of 24 kDa protein.

In uterine extracts a 28 kDa protein was present that was not detectable in the serum from the same animals. The IGFBPs with molecular masses of 38–42, 30–32 and 24 kDa were detected in the uterine extracts and appear to be similar in size to those in the serum (Fig. 1). The 38–42 kDa proteins in uterine extracts in all probability represent IGFBP-3 proteins. The 24 kDa protein may represent IGFBP-4, but 28 and 30–32 kDa proteins remain to be characterized. The immunological characterization of these various binding proteins must await the availability of specific antibodies to these proteins in rats.

Regulation by growth hormone and oestradiol

Hypophysectomy caused a marked decrease in the 38–42 kDa proteins, the 30–32 kDa proteins and very little change, if any, in the 24-kDa protein in rat serum (Fig. 1a: lanes 1 and 3, Table 1). Similar results have been reported by Yang *et al.* (1989). Growth hormone treatment partially restored the decreased 38–42 and 30–32 kDa proteins. Similarly, in the uterine extracts, hypophysectomy caused a precipitous decrease in the 38–42 and 30–32 kDa proteins and growth hormone partially restored both 38–42 and 30–32 kDa proteins in uterine extracts (Fig. 1, Table 1). The 28 kDa protein in the uterine extract, on the other hand, was not altered by hypophysectomy or growth hormone treatment. The amount of 24 kDa protein in both uterine extracts and serum is not altered by the growth hormone status of the rats.

Oestradiol, at 1 µg per rat, caused more than a 50% decrease in both 38–42 and 28 kDa proteins and no change in 24 kDa protein in the uterine extracts of ovariectomized rats (Fig. 1;

Table 1). These effects of oestradiol were detected irrespective of the growth hormone status in these animals, indicating that oestradiol may induce downregulation of both 38–42 and 28 kDa IGFBP proteins. However, oestradiol induced an apparent increase (100%) in 30–32 kDa protein in both uterine extracts and serum of hypophysectomized–ovariectomized rats but not in pituitary-intact or growth hormone-treated hypophysectomized–ovariectomized rats. Total IGFBP activity, as determined by adding the BP activity in all IGFBP bands from each rat uterus, in the oestradiol-treated rat uterine extracts, irrespective of growth hormone status, was 30–55% lower than that in rats not receiving oestradiol (Table 1). These effects of oestradiol on IGFBPs appeared to be specific to the uterus, as these effects were not apparent in serum.

Discussion

In this study, we examined rat uterine extracts for the presence of IGFBPs. Western ligand blotting analysis revealed several bands of specific IGFBP species with molecular masses of 38–42, 30–32, 28 and 24 kDa; the 28 kDa protein was detected in uterine extracts but not in serum. The 38–42, 30–32 and 24 kDa proteins in the uterine extracts were similar in size to those in serum. Although the nature and degree of relatedness between the IGFBP species in these studies remains unclear, size considerations alone would suggest that the 28, 30–32, 38–42 and 24 kDa proteins correspond to IGFBP-1, -2, -3 and -4, respectively. The resolution of the exact nature of the 28 kDa protein is more difficult from these studies, as glycosylated IGFBP-4, IGFBP-5, glycosylated IGFBP-6 and a fragment of IGFBP-3 all run in a similar region on the gel. Complete characterization of these rodent IGFBPs in the uterus must await the availability of appropriate reagents.

IGFBPs were considered to act as carriers for IGFs and to protect IGFs from degradation, thereby ensuring sufficient IGF supply to target tissues. The basis for this concept was that IGFs and IGFBPs were thought to be synthesized only by the liver. It is now evident that both IGFs and IGFBPs are produced by many tissues (Rutanen *et al.*, 1986; Pekonen *et al.*, 1988; Julkunen *et al.*, 1989; Giudice *et al.*, 1991). Our results extend these studies and further demonstrate that IGFBPs are extracted from rat uterus. Although the possible contribution of blood IGFBPs to that in uterine extracts cannot be excluded completely, this possibility is less likely because (1) uteri were perfused and rinsed thoroughly (Van Wyk, 1985) (2) the blood contamination in the extracts was less than 0.001% based on the haemoglobin assay (Hardouin *et al.*, 1987) and (3) a substantial amount of 28 kDa protein was obtained from uterine extracts while this protein was undetectable in serum (Binoux *et al.*, 1986). We, therefore consider that these IGFBPs are produced locally and may contribute to the autocrine or paracrine modulation of IGF action in the uterus.

The observations reported here document the ability of GH to regulate 38–42 kDa proteins both in uterine extracts and in serum. However, GH was without significant effect on 24 kDa proteins in both serum and uterine extracts. These observations are in line with several studies demonstrating GH regulation of various IGFBPs (Clemmons *et al.*, 1989; Glasscock *et al.*, 1990). Growth hormone may modulate these uterine IGFBPs through its direct effects or indirectly acting through IGF-I, as has been described for some other growth hormone effects on IGFBPs. IGFBP-1 mRNA was detected in uterine epithelial cells and stromal glands and its expression during the process of decidualization was independent of the pituitary (Croze *et al.*, 1990). Our observation that the 28 kDa protein in the uterine extracts was not altered by GH status is in agreement with these studies.

The ability of oestradiol to reduce 28 and 38–42 kDa proteins in the rat uterine extract adds another dimension to the complexity of the role of this steroid in uterine physiology, as previous studies have demonstrated that uterine IGF-I and IGF-I receptors are regulated by oestrogens (Murphy *et al.*, 1988; Chandrasekhar *et al.*, 1990; Murphy, 1991). An increase in the abundance of IGFBP-1 protein has been reported in the luteal phase of the menstrual cycle (Rutanen *et al.*, 1986; Julkunen *et al.*, 1989) in human decidual tissue (Julkunen *et al.*, 1989; Rutanen *et al.*, 1985) and during the first half of pregnancy (Rutanen *et al.*, 1982; Bell and Smith, 1988). These reports together with our studies suggest that the IGFBP-1 upregulation may be progesterone dependent, whereas its downregulation appears to be oestrogen dependent.

Our studies of oestrogen downregulation of 28 kDa protein in the uterus of adult rats are in line with those reported for immature rats in which oestrogen reduced the IGFBP-1 mRNA abundance (Murphy, 1991). In addition, 38–42 kDa proteins were also reduced by oestrogens suggesting that oestrogens have regulatory effects on uterine IGFBPs. Although controversial, most of the data suggest that IGFBPs inhibit the biological actions of the IGFs. The IGF-I variants that have negligible affinity for IGFBPs are considerably more potent than IGF-I in a variety of cell culture systems (Sara and Hall, 1990). It is therefore possible that some of the oestrogen induced effects in the uterus are mediated through a decrease in IGFBP, in addition to increased IGF-I expression (Murphy, 1991), thus

increasing the bioavailability and functional activity of uterine IGF-I.

In conclusion, we have demonstrated the presence of several IGFBPs in the rat uterine extracts. The 28 kDa protein that was not detected in the serum was present in uterine extracts. Both growth hormone and oestrogens modulate the uterine IGFBP activity. Our present data that showed that oestrogens reduced uterine IGFBP activity demonstrates a modulatory role of this steroid on another component of the IGF system in this tissue. Previous studies have demonstrated the influence of oestrogens on uterine IGF-I and IGF-I receptors.

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