J.Serb.Chem.Soc. *68*(11)811–818(2003)
JSCS – 3101

UDC 577.175.72:576.314:66.067+541–148
Original scientific paper

Membrane-associated insulin-like growth factor (IGF) binding structures in placental cells

ROMANA MASNIKOSA, J. ANNA NIKOLIĆ and OLGICA NEDIĆ[#]

INEP – Institute for the Application of Nuclear Energy, Banatska 31 B, 11080 Zemun-Belgrade, Serbia and Montenegro

(Received 21 February, revised 20 June 2003)

Abstract: The biological activities of IGF-I and –II are mediated mainly by the type 1 IGF receptor (IGF 1R) and controlled by their interaction with soluble proteins, the IGF binding proteins (IGFBPs). Although there is a growing body of evidence that some IGFBPs may be cell surface-bound, published data concerning cell association of IGFBP-1 are scarce and none of them concern placental cells. The cell membranes used in this study were isolated from term human placentae. Detergent-solubilized membranes were shown to contain two types of IGF binding structures that were separated by gel filtration on a Sephadex G-100 column. Proteins in the first peak were eluted at V_0 (M_r > 100 kD) and they bound IGF-I with greater specificity and affinity than IGF-II and insulin. Most likely, they represented the IGF 1R. Small proteins ($M_r \approx$ 45 kD) were eluted with the membrane proteins in the second maximum. They were able to bind IGF-I and IGF-II, but not insulin. The identity of these proteins was shown to be IGFBP-1 on the basis of their reaction with specific anti-IGFBP-1 antibodies. To the best of our knowledge, the existence of IGFBP-1 associated with human placental cell membranes has not been reported in the literature before. Colocalisation of IGFBP-1 with IGF 1R in cell membranes could provide efficient modulation of IGF 1R receptor-ligand interactions.

Keywords: IGF-I, IGF-II, IGF 1R, IGFBP-1, placental cell membranes, gel filtration.

INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, are polypeptides with structural homology to proinsulin, affecting growth, differentiation, and various metabolic processes in an endocrine and autocrine/paracrine fashion. The signalling of both IGFs is mediated by the type 1 IGF receptor (IGR 1R), a heterotetrameric glycoprotein composed of two ligand-binding α-subunits and two transmembrane β-subunits (M_r 350 kD). It shares structural and functional homology with the well-characterized insulin receptor.[1] The IGF 1R binds IGF-I with high affinity, IGF-II with several fold lower affinity and insulin with more than 100-fold lower affinity.[2] IGF-II also binds to a type 2 IGF receptor (IGF 2R), a single-chain membrane-spanning glycoprotein (M_r 300 kD), which is selective for IGF-II and has no affinity for insulin. Its role in IGF-II signal transduction is still a matter of debate.[3]

Serbian Chemical Society active member.

Finally, the actions of the IGFs are modulated by a superfamily of six IGF-binding proteins (IGFBPs), IGFBP-1 through IGFBP-6, and a growing number of related proteins (IGFBP-rPs) whose physiological relevance has yet to be established. IGFBPs bind the IGFs with equal or even greater affinities than do the IGF receptors, and therefore are placed in a critical regulatory position between IGFs and their cell surface receptors. While some IGFBPs have been shown to inhibit IGF actions by preventing them from gaining access to the IGF receptors, others potentiate IGF actions by facilitating the ligand-receptor interaction.[4] Recent studies indicated that some IGFBPs have their own receptors that mediate IGF-independent actions. IGFBP-1 and IGFBP-2 contain intergrin recognition sequences, Arg-Gly-Asp (RGD), which is the minimum requirement for interaction with integrins on the cell surface. IGFBP-3 was shown to possess its own receptor and binds to heparin.[5]

The aim of this work was to investigate the membrane-associated IGF binding structures in human placental cell membranes.

EXPERIMENTAL

Materials

Sephadex G-100 was obtained from Pharmacia Biotech AB, Sweden. Human IGF-I and IGF-II were from ICN Biomedicals, USA. Porcine insulin was from Novo Research Institute, Denmark. $Na^{125}I$ was supplied by Polatom, Poland. Goat anti-hIGFBP-1 antiserum was obtained from Diagnostic Systems Laboratories Inc., USA. All other employed chemicals were purchased from Sigma Chemicals, USA.

Tracer

IGF-I, IGF-II and insulin were iodinated by the chloramine T method.[6] The specific activities of the ^{125}I-labelled peptides were approximately 100 μCi/μg.

Preparation of placental membranes

Placental membranes were prepared by a modification of the procedure given by Perdue *et al.*[7] Term human placenta was collected immediately after delivery, placed in ice-cold 50 mM phosphate buffered saline (pH 7.5) and washed extensively with the same buffer. The placenta was dissected free from large vessels, amniotic and chorionic membranes. The remaining tissue was minced and homogenized in a polytron homogenizer with 5 volumes of 50 mM HEPES buffer (pH 7.5) containing 250 mM sucrose, 1 mM EDTA and 2 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 600 × g for 15 min. The pellet was discarded and the supernatant centrifuged at 18 000 × g for 30 min. The supernatant obtained was further centrifuged at 100 000 × g for 60 min to yield a pellet designated as placental membranes. The crude membrane pellet was washed twice and finally resuspended in membrane buffer (50 mM HEPES, pH 7.5, containing 4 mM $MgCl_2$). Aliquots were stored frozen at – 80 ºC.

Solubilization of placental membranes

Membranes (20 mg of membrane proteins) were solubilized for 2 h at 4 ºC in 5 mL of 2 % Triton X-100 in membrane buffer. After centrifugation at 100 000 × g for 90 min, the insoluble pellet was discarded and the clarified extract (solubilizate) was divided into aliquots and stored frozen at –80 ºC until use.

The membrane protein concentration was determined by the method of Lowry,[8] and in Triton solubilizates by the method of Bradford.[9]

Gel filtration

Solubilized membrane proteins (1 mg) were incubated with 0.5 pmol ^{125}I-IGF-I (500 000 cpm) in the presence or absence of unlabelled ligands (65 pmol IGF-I, or 65 pmol IGF-II or 170 nmol insulin) at 4 ºC

overnight and chromatographed in 50 mM sodium phosphate buffer containing 100 mM NaCl and 0.1 % Triton X-100 (pH 7.5) on a Sephadex G-100 column (1.8 × 60 cm). The flow rate was 20 mL/h. 1 mL fractions were collected and their radioactivity (cpm) measured in a γ-counter (APEX Auto Gamma Counter, Micromedic Systems Inc.). The same experiments were performed with ^{125}I-IGF-II and ^{125}I-insulin as tracers. The column was calibrated using Blue Dextran 2000, egg albumin (EA, 45 kD) and IGF-I (7.5 kD).

Gel filtration was also used for the separation of the IGF binding structures. The solubilizate (4 mg of membrane proteins) was chromatographed without tracer and the proteins eluting between fractions 36 and 46 were collected and designated "peak 1 preparation", while those eluting between fractions 52 and 72 were designated "peak 2 preparation". The proteins present in peaks 1 and 2 were concentrated 2-5-fold and stored at 4 ºC. The proteins from the peak 1 preparation were used in competitive ligand binding assays, while the proteins from the peak 2 preparation were used in immunoaffinity interactions. The proteins from the peak 2 preparation (0.5 mL) were incubated with 0.5 pmol ^{125}I-IGF-I (or ^{125}I-IGF-II) in the presence or absence of goat anti-IGFBP-1 antiserum at 4 ºC overnight and analysed by gel filtration on the Sephadex G-100 column as described.

Competitive ligand binding assays

100 μg of membrane proteins (from placental membranes or solubilizate) or 0.1 mL of proteins from the peak 1 preparation were incubated with 17 fmol ^{125}I-IGF-I or –II (≈ 20 000 cpm) and increasing concentrations of unlabelled ligands (IGF-I, -II or insulin) in a final volume of 0.5 mL of membrane buffer containing 2 mg/mL BSA, at 4 ºC overnight. Triton X-100 at a final concentration of 0.04 % was present in all binding assays. Following incubation, 1.5 mL of 20 % (w/v) polyethylene glycol 6000 (PEG) was added. The tubes were vortex-mixed vigorously, left at 4 ºC for 20 min, then centrifuged (3000 × g, for 40 min) and the supernatants were discarded. Precipitated radioactivity was measured in the γ-counter. Nonspecific binding (NSB) was determined by incubating ^{125}I-IGF with binding buffer without membrane proteins. The specific binding was calculated as the difference between the total binding and the NSB.[10] The data were expressed as IC_{50} (concentration of the competing ligand that inhibited labelled ligand-specific binding by 50 %) and analysed by the method of Scatchard.[11]

RESULTS

Detergent solubilized IGF-I binding structures from placental cell membranes were resolved into two components on a Sephadex G-100 column (Fig. 1a). The first peak, designated peak 1, appeared at an elution volume (V_e) of 41 mL which was equivalent to the void volume of the column, V_o. The second peak, designated peak 2, eluted at V_e of 62 mL, which is the V_e of egg albumin, indicating that proteins in this peak had a molecular weight of approximately 45 kD. Proteins in the second maximum bound approximately three times more ^{125}I-IGF-I than proteins in the first peak. The third radioactive maximum in the elution profile ($V_e \approx 99$ mL) represented unbound ^{125}I-IGF-I. (Fig. 1a)

The ^{125}I-IGF-I binding to the proteins present in peaks 1 and 2 was specific, as both peaks disappeared upon gel filtration in the presence of 65 pmol of unlabelled IGF-I. IGF-II was an equally potent inhibitor of the ^{125}I-IGF-I binding (results not shown), while insulin at a 2000-fold greater concentration could displace only about 30 % of the radioactivity initially bound in peak 1 (Fig. 1b). This pattern of crossreactivity is characteristic of placental IGF 1R and it suggested the presence of this receptor in peak 1.[12] Proteins in peak 2, however, bound more ^{125}I-IGF-I in the presence of insulin than in its absence (Fig. 1b). It is probable that insulin occupied a proportion of the IGF-I binding sites on IGF 1R, allowing ^{125}I-IGF-I to bind to a greater extent to proteins in peak 2.

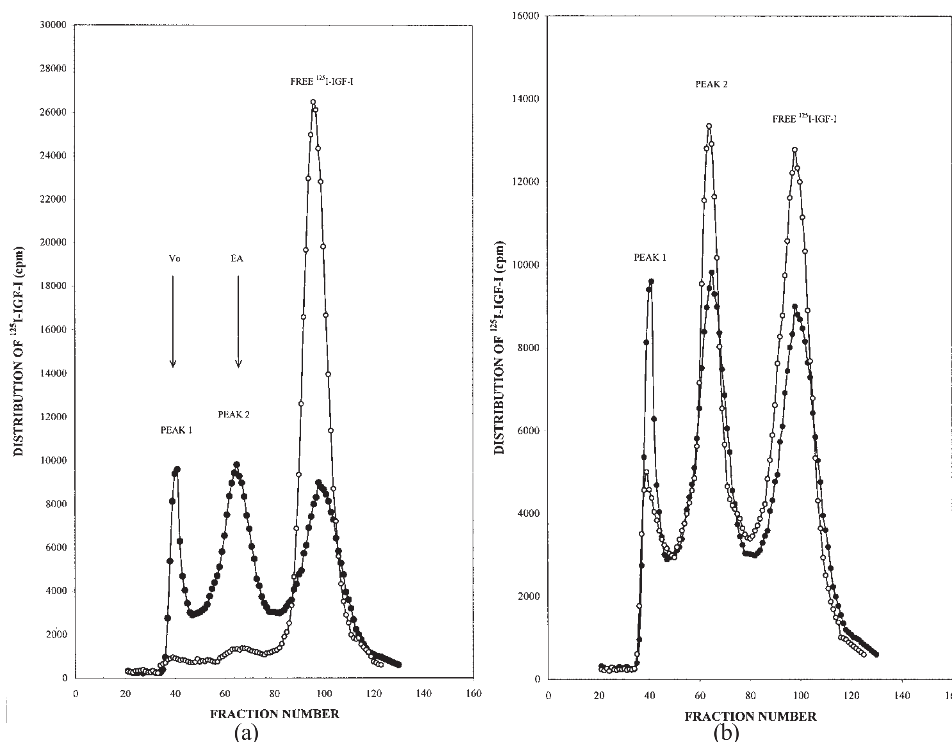

Fig. 1. Gel filtration of solubilized placental membrane proteins, preincubated with ^{125}I-IGF-I, without competing ligand (●) or in the presence of unlabelled competitor (○): a) IGF-I; b) insulin.

As decidua is the primary source of IGFBP-1 in pregnant women[13] and as IGFBP-1 is the predominant IGFBP in amniotic fluid,[14] the interaction between specific anti-IGFBP-1 antibodies and proteins in peak 2 was tested. Proteins in peak 2 were separated by gel filtration, equilibrated with ^{125}I-IGF-I in the presence or absence of anti-IGFBP-1 antibodies and, again, analysed by gel filtration. The elution profiles obtained are given in Fig. 2. In the presence of anti-IGFBP-1 antibodies the peak at a V_e of 62 mL disappeared from the elution profile and a new peak, expected to represent the ^{125}I-IGF-I/IGFBP-1/anti-IGFBP-1 antibody complex, appeared at V_o. The experiment was also performed with ^{125}I-IGF-II as a tracer and similar elution profiles were obtained, while ^{125}I-insulin did not interact at all (data not shown). These results strongly indicated the presence of IGFBP-1 in the placental membranes. The possibility that peak 2 reflected the presence of contaminating soluble IGFBP-1 was ruled out, since repeated washing of the membranes prior to solubilization did not alter the magnitude of the peak.

The displacement of ^{125}I-IGF-I from crude placental membranes, membrane solubilizates or proteins in peak 1, separated by gel filtration, by competitive binding assays with unlabelled IGF-I, IGF-II and insulin was examined and the IC_{50} values obtained are summarized in Table I. The pattern of crossreactivity was again characteristic of the IGF

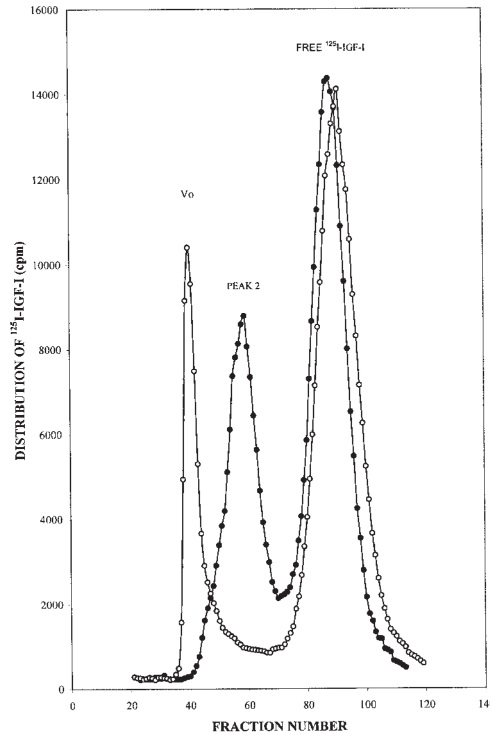

Fig. 2. Gel filtration of the proteins in peak 2, preincubated with ^{125}I-IGF-I in the absence (●) or in the presence of anti-IGFBP-1 antibodies (○).

1R: IGF-I > IGF-II >> insulin.[12] Moreover, each successive step in the IGF 1R separation (membrane solubilization and chromatographic separation of IGF 1R) caused the IC_{50} values for all unlabelled ligands to decrease.

TABLE I. Binding of ^{125}I-IGF-I to crude placental membranes, solubilized membranes and proteins in peak 1 separated by gel filtration in competition with the unlabelled ligands: IGF-I, IGF-II and insulin

	IC_{50} (nM)		
	IGF-I	IGF-II	INSULIN
Crude membranes	0.4	0.8	1000
Solubilizates	0.1	0.6	72
Peak 1 proteins	0.05	0.3	8.3

The IC_{50} values obtained could be attributed to the interaction of ligands with the IGF 1R, because IGFBP-1/^{125}I-IGF-I complexes were not precipitated by PEG (results not shown). Scatchard analysis of the IGF-I binding to solubilized membrane proteins resulted in a curvilinear plot, providing evidence that both high affinity and low affinity binding sites existed (Fig. 3). The dissociation constant (K_d) that characterized high affinity interaction was estimated to be 0.08 nM, and the binding capacity (R_o) was approximately 0.05 pmol/mg membrane protein. The slope of the curve representing the low affinity binding system was very flat on the Scatchard plot and could not be clearly distinguished

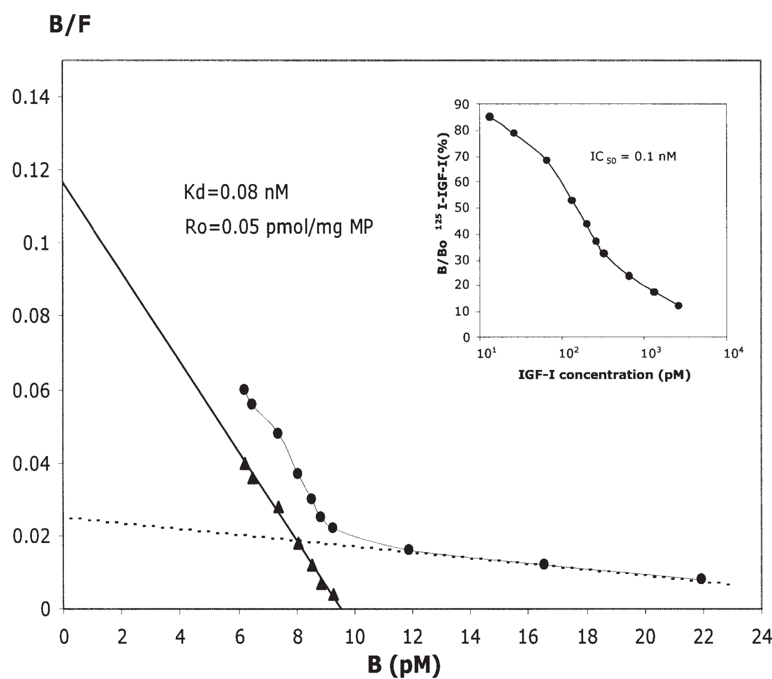

Fig. 3. Scatchard plot of IGF-I binding to placental membrane solubilizates. The ordinate represents the ratio of bound to free IGF-I (B/F). The contribution of the low affinity binding sites was extrapolated (–––) and subtracted from the total binding (●) to yield the line representing the high affinity binding system (▲). The displacement of ^{125}I-IGF-I bound to membrane solubilizate by increasing concentrations of IGF-I is inserted as a curve (●).

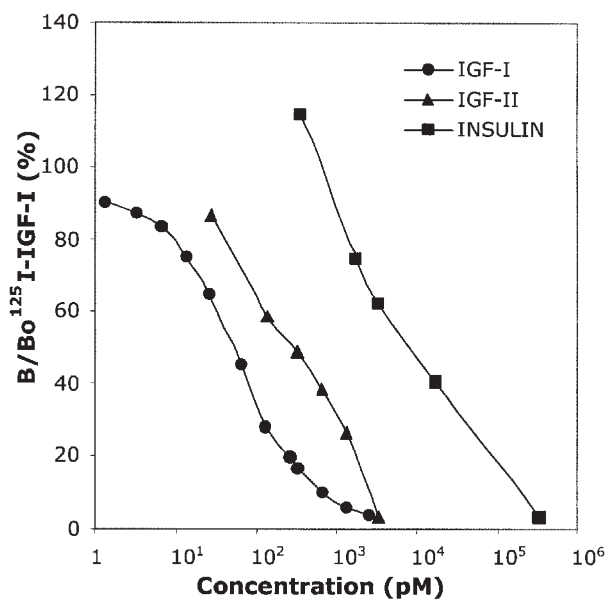

Fig. 4. Inhibition of ^{125}I-IGF-I binding to proteins of peak 1 by increasing concentrations of IGF-I (●), IGF-II (▲) and insulin (■). Data are expressed as the percentage of maximal specific binding in the absence of unlabelled ligand. Each point represents the mean of two independent determinations.

from nonspecific binding. In binding studies using ^{125}I-IGF-I and proteins in peak 1 (IGF 1R), insulin in low doses caused enhancement of ^{125}I-IGF-I binding to IGF 1R compared to the degree of binding in the absence of this competitor, demonstrating that the affinity of IGF 1R is influenced by interaction with other proteins (Fig. 4).

DISCUSSION

Two IGF-I binding species were detected in human placental cell membranes and solubilizates and separated by gel filtration. The first IGF-I binding structure bound IGF-I with high affinity (IC_{50} = 0.05). The specific interaction could be inhibited by IGF-II and insulin in a pattern characteristic of placental IGF 1R.[12] The second site of ^{125}I-IGF-I (and ^{125}I-IGF-II) binding was shown to be IGFBP-1. Widely recognised as soluble proteins, IGFBPs have been recently shown to associate with cell surfaces and components of the extracellular matrix.[5] Specific binding of IGFBP-1 to the $\alpha 5\beta 1$ integrin (fibronectin receptor) in Chinese hamster ovary cells has been demonstrated.[15] In this study evidence is provided that IGFBP-1 is associated with human placental cell membranes. However, it is premature to speculate about the nature of this association.

It has recently been postulated that IGFBP-1 is a key regulator of IGF-I activity within the local environment of the placenta.[16] IGFBP-1 is synthesised in decidual cells, while the highest levels occur in amniotic fluid.[13,14] Therefore, it is reasonable to speculate that decidual IGFBP-1 is transported through the trophoblast cells of the placenta to the amniotic fluid. The mechanism of transport is still unknown.

It has been noted in this study that IGF-1, IGF-II and insulin bind with higher affinity to solubilized receptors compared to crude membranes and that the proteins in peak 1 (presumed to be IGF 1R) exhibited an even greater difference in their binding properties when separated from IGFBP-1 by gel filtration. One possible explanation of this phenomenon would be a conformational change in the receptor structure, due to the artificial experimental milieu, which resulted in a high-affinity state of the IGF 1R.[17] It should be noted that the insulin receptor does not exhibit a comparable change in properties following solubilization.[18]

Data from our laboratory support an alternative hypothesis, that a competitive binder, IGFBP-1, positioned in the proximity of IGF 1R, in the placental cell membranes, regulates the affinity of IGF 1R for its ligands. The concept of an affinity regulator, whose activity might be modulated by physiological circumstances, although attractive, needs further investigation.

Acknowledgements: This work was supported by the Ministry of Science, Technology and Development of Serbia, project 1504: Glycobiological aspects of physiological and pathophysiological processes.

ИЗВОД

ВЕЗУЈУЋЕ СТРУКТУРЕ ЗА ИНСУЛИНУ СЛИЧНЕ ФАКТОРЕ РАСТА (IGF) У МЕМБРАНАМА ПЛАЦЕНТАЛНИХ ЋЕЛИЈА

РОМАНА МАСНИКОСА, Џ. АНА НИКОЛИЋ и ОЛГИЦА НЕДИЋ

ИНЕП – Институт за примену нукеларне енергије, Банатска 31 Б, 11080, Београд-Земун

IGF-I и IGF-II постижу своје биолошке ефекте првенствено путем везивања за тип 1 IGF рецептора (IGF 1R), а у интеракцији између ових пептида и рецептора посредују специфични растворни протеини, IGF везујући протеини (IGFBPs). У литератури је све више података о везивању појединих IGFBP за ћелијске површине, мада је мало радова који се односе на везивање IGFBP-1 за ћелије. Ћелијске мембране, које су коришћене у овом раду, изоловане су из ткива терминске хумане плаценте. Након солубилизације мембрана, гел-филтрацијом на Sephadex-у G-100 раздвојене су две врсте протеина које везују IGF молекуле. Протеини у првом максимуму, елуирани у нултој запремини колоне (V_o), везивали су IGF-I са већом специфичношћу и афинитетом у поређењу са IGF-II и инсулином и највероватније представљају IGF 1R. У оквиру мембранских протеина елуираних у другом максимуму детектовани су мањи протеини ($M_r \approx 45$ kD), који су везивали IGF-I и IGF-II, али не и инсулин. На основу њихове реактивности са анти-IGFBP-1 антителима, утврђено је да се ради о IGFBP-1. Колико је нама познато, овај рад представља прву потврду присуства IGFBP-1 у мембранама плацентних ћелија. Присуство молекула IGFBP-1 у близини мембрански усидреног IGF 1R могло би обезбедити ефикасну модулацију интеракција између рецептора и одговарајућег лиганда.

(Примљено 21 фебруара, ревидирано 20 јуна 2003)

REFERENCES

1. D. LeRoith,. H. Werner, D. Beitner-Johnson, C. T. Roberts, *Endocr. Rev.* **16** (1995) 143
2. C. E. H. Stewart, P. Rotwein, *Physiol. Rev.* **76** (1996) 1005
3. S. Kornfeld, *Annu. Rev. Biochem.* **61** (1992) 307
4. J. L Martin, R. C. Baxter, in *The IGF System*, Humana Press, New Jersey, 1999, p. 227
5. O. Youngman, R. G. Rosenfeld, in *The IGF System*, Humana Press, New Jersey, 1999, p. 257
6. J. A. Nikolić, M. Ratković, O. Nedić, *J. Serb. Chem. Soc.* **61** (1996) 1149
7. J. F. Perdue, J. K. Chan, C. Thibault, P. Radaj, B. Mills, W. H. Daughaday, *J. Biol. Chem.* **258** (1983) 7800
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193** (1951) 265
9. M. M. Bradford, *Anal. Biochem.* **72** (1976) 248
10. W. H. Daughaday, I. K. Mariz, B. Trivedi, *J. Clin. Endocrinol. Metab.* **53** (1981) 282
11. G. Scatchard, *Ann. N. Y. Acad. Sci.* **51** (1949) 660
12. A. E. Bennett, R. L. Hintz, *Horm. Metab. Res.* **18** (1986) 49
13. E. M. Rutanen, M. Seppälä, *Int. J. Gynaecol. Obstet.* **39** (1992) 3
14. G. Póvoa, G. Enberg, H. Jörnvall, K. Hall, *Eur. J. Biochem.* **144** (1984) 199
15. J. I. Jones, A. Gockerman, W. H. Busby, G. Wright, D. R. Clemmons, *Proc. Natl. Acad. Sci. USA* **90** (1993) 10553
16. J. M. Gibson, J. D. Aplin, A. White, M. Westwood, *Mol. Hum. Reprod.* **7** (2001) 79
17. E. L. Germain-Lee, M. Janicot, R. Lammers, A. Ullrich, S. J. Casella, *Biochem. J.* **281** (1992) 413
18. K. Siddle, M. A. Soos, in *The IGF System*, Human Press, New Jersey, 1999, p. 207.