Lectin-induced alterations of the interaction of insulin and insulin-like growth factor 1 receptors with their ligands

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(Received 16 October 2007, revised 15 January 2008)

Abstract: In order to study whether the carbohydrate moieties of the human placental IGF-I receptor (IGF1R), IGF-II receptor (IGF2R) and insulin receptors (IRs) play a role in ligand binding, solubilised cell membrane preparations were incubated with 125I-labelled IGF-I, IGF-II and insulin in the presence of lectins with different sugar specificities. Three incubation procedures were tested: ligand-first, co-incubation and lectin-first incubation. Wheat germ agglutinin (WGA), concanavalin A (Con A) and phytohaemagglutinin (PHA) altered the binding of IGF-I and insulin to their high-affinity receptors in a lectin specific and dose-dependent manner, whereas these lectins did not affect the interaction of IGF-II with its receptor(s). Moreover, the same lectins either inhibited or enhanced IGF-I and insulin binding, depending on the incubation scheme. These results also suggest that IR-A and IR-B from human placenta might be differently glycosylated.

Keywords: IGF1R; IGF2R; IR; WGA; Con A; PHA.

INTRODUCTION

The insulin-like growth factor (IGF) system is a complex assemblage of peptide hormones (IGF-I and IGF-II), receptors and binding proteins.1 The peptides IGF-I and IGF-II (IGFs) bind to the insulin/IGF family of cell surface receptors, namely, the insulin-like growth factor I receptor (IGF1R) and insulin receptors (IRs), and activate their intrinsic tyrosine kinase domains. These activated receptors initiate signalling cascades that ultimately result in the regulation of a number of biological responses.1 The components of the IGF system act together to control several crucial biological outcomes, including cellular growth, proliferation, differentiation, survival against apoptosis and migration.1,2

The type 2 IGF receptor (IGF2R) is structurally dissimilar to IGF1R and IR, has no intrinsic signalling transduction capability and, in the context of the IGF

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doi: 10.2298/JSC0809793M
system, primarily acts to sequester IGF-II from potential receptor activating interactions and to internalise and degrade IGF-II. The IGF2R binds IGF-II with a high affinity but binds IGF-I with a very low affinity and does not bind insulin.

It is known from earlier genetic experiments that IR mediates growth in response to IGF-II during foetal development of the mouse. The homodimeric IR exists in two isoforms, which arise from the alternative splicing of exon 11 in the IR mRNA. Exon 11 codes for twelve amino acids which are inserted upstream of the third last residue of the extracellular α-subunits of the IR-B isoform. The IR-A isoform lacks these twelve amino acids. The presence or absence of the exon 11-encoded peptide yields two receptors with unique biochemical properties. IR-A and IR-B seem to be localised on different regions of the plasma membrane. The IR-A has been identified as a high affinity receptor for IGF-II, which is preferentially expressed in foetal and cancer cells.

Genetic evidence strongly suggests different roles for IGF1R and IR, despite their overall structural homology. The two receptors activate common intracellular pathways. In spite of attempts to elucidate the molecular basis of IGF1R vs. IR action, it is unclear what determines the signalling specificity in vivo. Entingh-Pearsall and co-workers proposed that other factors could affect signalling, including the time course of stimulation of a cell with different ligands. It is also believed that other molecules can influence the binding kinetics of the receptors.

The hIR has eighteen potential sites for N-glycosylation, of which sixteen are glycosylated. The insulin binding α-subunit contains fourteen potential N-linked glycosylation sites. The receptor is heavily glycosylated, as 22% of its molecular mass is composed of carbohydrate. Multiple potential sites for N-linked glycosylation (sixteen) are also found in the hIGF1R molecule. IGF2R has nineteen potential N-glycosylation sites.

Recently, N-glycans attached to human placental IGF and insulin receptors were characterised and multiple populations of the receptors, which bore different sugars at their termini, were observed. The primary objective of the current study was to examine how lectins with different sugar specificities modify the interactions of IGFs and insulin with their glycoprotein receptors from human placenta.

**EXPERIMENTAL**

**Materials**

Porcine insulin was from Novo Nordisk (Bagsværd, Denmark). Human IGF-I and IGF-II were from GroPep Pty Ltd. (Adelaide, Australia). Hepes, poly(ethylene glycol) 8000 (PEG), bovine immunoglobulin G (IgG, technical grade), bovine serum albumin (BSA) and Triton X-100 were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Na125I for iodination was purchased from Isotope (Budapest, Hungary). Wheat germ agglutinin (WGA) was from Vector Laboratories (Burlingame, CA, USA). Concanavalin A (Con A) was from Amersham Biosciences (Little Chalfont, UK). The phytohaemagglutinin (PHA) used in this study (a mixture of E-PHA and L-PHA) was from INEP (Belgrade, Serbia).
Methods

$^{125}$I-IGF-I, $^{125}$I-IGF-II and $^{125}$I-insulin (tracers) were prepared using the chloramine T method. Iodination was performed every three weeks and resulted in specific activities of $1.0\text{ to }2.0\times10^8$ cpm µg$^{-1}$.

Human placental tissue was obtained from uncomplicated pregnancies at term, according to protocols approved by the local ethical committee. Solubilised placental membranes were prepared essentially as described previously. The protein concentration in the solubilised membranes was determined by the method of Bradford.

The lectins were dissolved according to the manufacturer’s instructions to give stock solutions of a final concentration of 5 mg ml$^{-1}$. WGA was dissolved in 0.010 M HEPES buffered saline (HBS), pH 7.5; Con A in 0.050 M phosphate buffered saline (PBS), pH 6.5 and PHA in PBS, pH 7.5. Serial lectin dilutions were prepared, ranging from 0 to 2310 nmol L$^{-1}$ for WGA, from 0 to 943 nmol L$^{-1}$ for Con A and from 0 to 870 nmol L$^{-1}$ for PHA in 0.050 M PBS buffer containing 0.25 % BSA.

The binding assays were performed with solubilised membranes diluted in assay buffer (0.050 M HBS, pH 7.5) to give a membrane protein concentration of 1.0 mg mL$^{-1}$. In the simplest lectin binding assays, solubilised membranes (100 µg of membrane protein per tube) were incubated with 0.10 pmol of $^{125}$I-IGF-I, $^{125}$I-IGF-II or $^{125}$I-insulin (10$^5$ cpm) in the absence or presence of WGA, Con A or PHA solution (final concentration 20 µg mL$^{-1}$) in a fixed reaction volume of 0.50 mL containing assay buffer with BSA (final concentration 1.2 %). The second set of assays employed solubilised membranes, $^{125}$I-ligands and increasing concentrations of plant lectins.

Three different incubation procedures were tested: 1) co-incubation, in which all three reactants (solubilised membranes, $^{125}$I-ligand and plant lectin) were added simultaneously to the test tubes and incubated for 24 h at 4 °C, 2) lectin-first incubation, in which the solubilised membranes were first incubated with a lectin for 2 h, and then $^{125}$I-ligand was added and 22 h at 4 °C was allowed for equilibration and 3) ligand-first incubation, in which the solubilised membranes were first incubated with $^{125}$I-ligand for 22 h at 4 °C, then a plant lectin was introduced followed by a further 2 h incubation.

After incubation, the receptor/radioligand complexes were precipitated by the addition of bovine IgG (final concentration 0.050 %) and 1.5 ml of PEG solution (20 % in 0.050 M PBS, pH 7.5) to each tube. The tubes were vortexed, centrifuged (4500 x g for 45 min) and the supernatants were aspirated off. The precipitated $^{125}$I-ligand radioactivity was measured in an automatic gamma counter (1470 Wallac Wizard, Perkin-Elmer, USA). Non-specific binding (NSB) was measured in reaction tubes which contained all reactants except solubilised membranes. The maximal binding ($B_0$), the quantity of $^{125}$I-ligand bound to the receptors in the absence of lectin, was expressed as the percentage of the total available $^{125}$I-ligand concentration ($T$). The specific binding in the presence of each lectin ($B$) was expressed as the percentage of that in its absence ($B/B_0\times100$). In each experiment, the specific binding was corrected for NSB. The data were plotted as a function of lectin concentration vs. $B/B_0$.

All curves were created and fitted using Origin Pro Version 7.5 (Origin Lab, Northampton, MA, USA). Statistical analyses were made using SPSS 10 software (SPSS Inc., Chicago, IL, USA). The $B/B_0$ data from the binding assays were subjected to one way Anova (with the independent variable being plant lectin or $^{125}$I-ligand) followed by the LSD test. $P < 0.05$ was considered significant.
RESULTS

The tracer quantities of the $^{125}\text{I}$-ligands used in this study corresponded to physiological concentrations of IGFs and insulin, thus ensuring that each ligand preferentially bound to the receptor of the highest affinity. The interactions of $^{125}\text{I}$-ligands with their receptors in the solubilised membranes were examined in the presence of increasing concentrations of lectins. The data obtained from the co-incubation experiments were grouped according to the employed $^{125}\text{I}$-ligand and are presented in Figs. 1A to 1C.

Maximal binding ($B_0$) was 9.6±1.0 % for $^{125}\text{I}$-IGF-I, 10.1±1.2 % for $^{125}\text{I}$-IGF-II and 15.4±0.9 % for $^{125}\text{I}$-insulin. The lectin dose-dependent binding curves for $^{125}\text{I}$-IGF-I (Fig. 1A) and $^{125}\text{I}$-insulin (Fig. 1C) were similarly shaped and they were mostly inhibitory throughout the examined lectin concentration range. The $^{125}\text{I}$-IGF-II binding curves (Fig. 1B) did not reflect great changes in the tracer binding as a function of lectin concentration.

The most profound inhibition of ligand binding was observed after co-incubation of $^{125}\text{I}$-insulin, IRs from solubilised membranes and WGA, when the $B/B_0$...
value decreased to 18.2±1.3 % (Fig. 1C). This resembled the degree of displacement of 125I-insulin bound to placental IRs by unlabelled insulin. At the largest employed concentration of lectin, Con A and PHA demonstrated weaker inhibitory effects on the 125I-insulin–IR interaction, than did WGA. The effect seemed to be receptor-specific, as the same lectins showed weaker inhibitory effects on 125I-IGF-I binding to IGF1R (Fig. 1A). Thus, WGA and PHA lowered the specific binding of 125I-IGF-I to 68.2±4.6 and 67.1±5.2 % of the initial values, respectively, whereas the specific binding of this tracer reached 78.6±5.1 % in the presence of the maximally effective Con A concentration.

In the second set of binding assays, the effect of three different types of incubation: ligand-first incubation, co-incubation and lectin-first incubation on the specific binding of 125I-labelled ligands to their receptors was examined. The final lectin concentration of 20 μg mL−1 was chosen as it initiated a significant inhibition of the 125I-ligand–receptor binding (the penultimate points on the graphs in Figs. 1A and 1C). The B/B₀ values obtained from three independent experiments performed in triplicate were subjected to statistical analysis. The results are given in Table I.

TABLE I. Effects of lectins on the displacement of 125I-ligand binding to solubilised placental cell membranes. Solubilised membranes (100 µg of membrane protein per tube) were incubated with 0.10 pmol of 125I-IGF-I, 125I-IGF-II or 125I-insulin (105 cpm) in the absence or presence of WGA, Con A or PHA solution (final concentration 20 µg mL⁻¹) in a fixed reaction volume of 0.50 ml containing assay buffer with BSA (final concentration 1.2 %). Specific binding in the presence of each lectin (B/B₀×100) is expressed as the percentage of that in its absence (no lectins present; the binding normalised to 100 %). The effect of three different types of incubation: ligand-first incubation, co-incubation and lectin-first incubation on the specific binding of 125I-labelled ligands to their receptors was tested. B/B₀ values obtained from three independent experiments performed in triplicate (means ±SD) were subjected to statistical analysis.

<table>
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<th>125I-Ligand</th>
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<th>Ligand-first</th>
<th>Co-incubation</th>
<th>Lectin-first</th>
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<?Significant at P ≤ 0.001; bnot significant, as compared to control binding (no lectins); csignificant at P < 0.05
The order of reagent addition substantially affected the specific binding of $^{125}$I-IGF-I and $^{125}$I-insulin (Figs. 2A and 2C), whereas the specific binding of $^{125}$I-IGF-II was, generally, not changed (Fig. 2B). The co-incubation and lectin-first incubation schemes resulted in inhibition of the specific binding of $^{125}$I-IGF-I and $^{125}$I-insulin. However, in the ligand-first incubation scheme, the binding of $^{125}$I-IGF-I and $^{125}$I-insulin presented completely different patterns, as inhibition was replaced by enhancement of the binding (Figs. 2A and 2C).

Fig. 2. Effect of lectins on $^{125}$I-ligand binding to solubilised placental cell membranes in three different types of incubation. Solubilised membrane proteins were incubated with $^{125}$I-IGF-I (A), $^{125}$I-IGF-II (B) or $^{125}$I-insulin (C) alone or with the lectin indicated (20 μg mL$^{-1}$ in the tube). Three different types of incubation were tested. The specific binding of $^{125}$I-ligands was determined by PEG precipitation assay as described in the "Experimental". Specific binding in the presence of lectin ($B$) was expressed as the percentage of that in its absence ($B_0$). The means ±SD of three independent experiments, performed in triplicate, are shown.

Compared to the control experiment (no lectins present; the binding normalised to 100 %), all three lectins provoked significant changes in the specific binding of $^{125}$I-IGF-I to its receptor ($P \leq 0.001$), the only exception being the combination of $^{125}$I-IGF-I and Con A in the ligand-first type of incubation (Table I). The most prominent inhibition of $^{125}$I-IGF-I binding to IGF1R occurred when PHA was added to the solubilised receptors prior to the radioligand ($B/B_0 = 53.9±4.9$ %).

In general, the binding of $^{125}$I-IGF-II to its placental receptor(s) was not changed in the presence of lectins (Fig. 2B). The presence of WGA in co-incubation or PHA in the lectin-first incubation scheme was of minor statistical signi-
ficance ($0.001 < P < 0.05$, Table I), compared to the control binding. These findings led to the omission of $^{125}$I-IGF-II from the third set of experiments.

As for the $B/B_0$ values obtained with $^{125}$I-insulin, the presence of all three lectins potently altered the magnitude of the tracer binding. Of all the tested lectins, WGA exerted the greatest influence on $^{125}$I-insulin binding to the placental IRs, causing the corresponding $B/B_0$ values to increase to $167.8\pm3.8\%$ in the ligand-first type of incubation or to decrease to $35.6\pm3.3\%$ in the co-incubation assays (Table I).

When the lectin was set as the independent variable, statistical analysis of the binding data showed that WGA and PHA exerted different effects on $^{125}$I-ligand binding to their receptors, regardless of the type of incubation ($P \leq 0.001$). Statistically significant differences between the binding of $^{125}$I-IGF-I and $^{125}$I-IGF-II to the placental receptors were not observed in the presence of Con A in the ligand-first ($P = 0.752$) and lectin-first type of incubation ($P = 0.740$). The binding of $^{125}$I-IGF-I and $^{125}$I-insulin was not different in the presence of the same lectin ($P = 0.549$).

In the third set of experiments, the interactions of $^{125}$I-IGF-I and $^{125}$I-insulin with their receptors from the solubilised membranes were examined in the presence of increasing concentrations of lectins in two different incubation schemes, i.e., ligand-first and lectin-first incubation. The obtained data are presented in Figs. 3A and 3B.

Fig. 3. Effect of lectins on the binding of $^{125}$I-IGF-I and $^{125}$I-insulin to solubilised placental membranes. Solubilised placental membranes were incubated with $^{125}$I-IGF-I (A) or $^{125}$I-insulin (B) and increasing concentrations of WGA, Con A or PHA. Ligand-first and lectin-first incubation schemes are shown. See legend for Fig. 1.

The binding curves obtained for the two radioligands were similar in shape for each lectin, both for the ligand-first and the lectin-first incubation scheme. The lectin-first incubation curves lay mostly in the inhibitory range, whereas their ligand-first counterparts lay mostly in the "activatory" range (Figs. 3A and 3B).
The \( B/B_0 \) values of \(^{125}\text{I}-\text{IGF-I}\) binding in ligand-first incubation with Con A were close to 100% throughout the studied lectin concentration range (Fig. 3A), suggesting that this lectin had no influence on the interaction of the tracer with IGF1R. These data confirmed those obtained in the binding assays using a fixed concentration of lectin (Fig. 2A). The statistical analysis also showed that Con A had no significant effect on the interaction of \(^{125}\text{I}-\text{IGF-I}\) with the placental IGF1R (Table I). As for \(^{125}\text{I}-\text{insulin}\), all lectins tested in the lectin-first type of incubation displaced the tracer bound to the IR and caused the \( B/B_0 \) values to decrease significantly, but not until a concentration of 10 \( \mu g \) mL\(^{-1} \) was attained (Fig. 3B).

In general, the experiments described above suggest that the affinity of the solubilised placental IGF1R and IR towards their ligands was affected by interaction with plant lectins.

**DISCUSSION**

IGF1R and IR share a high degree of homology. Their functions are physiologically distinct but overlapping.\(^9\) Each receptor, however, encroaches on the others domain, suggesting that they have an intrinsic ability to mediate other functions.\(^9\) Some investigators believe that the differences between IGF1R and IR can be ascribed to extrinsic factors\(^19\) or that the cellular environment may alter signalling.\(^11\) Thus the search for putative affinity modulators that can bind to IGFR and IR is becoming increasingly attractive.

In a previous study, the differences in glycosylation between IGFRs and IR from human placenta were analysed.\(^16\) The pattern of binding to five different immobilised lectins indicated that the glycosylation of these receptors differed. Several populations of the receptors were found, with various types of sugar branches. In the work presented here, binding assays were used to characterise more extensively the interactions of the oligosaccharide branches of IGFRs and IR of human placenta with lectins.

It was observed that WGA, Con A and PHA caused the specific binding of \(^{125}\text{I}-\text{IGF-I}\) and \(^{125}\text{I}-\text{insulin}\) to change in a lectin specific and dose-dependent manner, whereas the same lectins did not alter the binding of \(^{125}\text{I}-\text{IGF-II}\) to its receptor(s). The effect of lectins on the binding of \(^{125}\text{I}\)-labelled peptides to their receptors was ligand specific, as concluded after careful statistical analysis of the binding data. In other words, IGF1R, \(^{125}\text{I}-\text{IGF-II}\) binding receptor(s) and IR responded differently to particular plant lectins. The same lectins were able either to inhibit or enhance the specific binding, depending on the scheme of incubation. Inhibition of \(^{125}\text{I}\)-ligand binding to solubilised placental IGF1R or IR occurred when the lectin was added to IGF1R or IR either simultaneously or before the ligand. Enhancement of the specific binding occurred when the lectins were added after the ligand had been equilibrated with its receptor.
The inhibition, most probably, originated from an overlapping of the ligand binding sites and the lectin binding sites on the receptors or because of a conformational change in the receptor molecule after lectin binding. WGA provoked the most prominent inhibition of insulin binding to IR in the present experimental system. This lectin promotes insulin-mimetic effects. The strongest inhibition of $^{125}$I-IGF-I binding to IGF1R was observed when PHA was added to the receptor prior to the tracer. Given the sugar binding specificity of WGA and PHA, their potent inhibitory effects may be attributed to the interaction with complex type $N$-glycans, with repeating $N$-acetyllactosamines at their termini, attached to the molecules of IGF1R and IR. These results are consistent with those published previously, where strong adsorption of $^{125}$I-IGF-I/IGF1R complexes and $^{125}$I-insulin/IR complexes to agarose-immobilised WGA and PHA was found. Poly-$N$-acetyllactosamine chains were detected in the carbohydrate branches of the human lymphocyte IR. Moreover, both high-mannose type and bi-, tri- and tetra-antennary complex type oligosaccharide structures were recently found on IR molecules from mouse liver. Despite these findings, the roles of the saccharide chains in the functions and metabolism of the IR are not fully elucidated. The types of glycans attached to IGF1R have so far received little attention of researchers.

The first three domains of the IR $\alpha$-subunit differ from those of the IGF1R in the sequences governing ligand specificity. $N$-Linked carbohydrates are attached at the Asn residues 16, 25, 111, 215, 255, 337, 397 and 418 in the L1-CR-L2 fragment of IR. Interestingly, a residue analogous to Asn16 of the IR is missing in the sequence of the IGF1R, as is Asn255. IR residue Asn15 is one of the most important residues for the high-affinity binding of insulin. The high-affinity binding of IGF-I to IGF1R involves a specific sequence in the CR domain of the receptor, which is positioned seven residues away from a residue analogous to Asn255. Thus, the main differences in glycosylation between IGF1R and IR seem to lie near the amino acid sequences which participate in the high affinity ligand binding to the receptors. The different effects of the different plant lectins on the interactions of IGF-I and insulin with their receptors, observed here, can be explained in light of these data. This might also suggest a role for the saccharides attached to IGF1R and IR in interactions with other proteins, possibly placental lectins.

The alteration of the specific binding of certain hormones and growth factors to their receptors in the presence of plant lectins has already been described in the literature. Thus, Vale and Shooter reported that of eight tested plant lectins, only WGA significantly inhibited (50 %) the binding of $^{125}$I-labelled nerve growth factor (NGF) to cells. Buxser and co-workers described increased binding of $^{125}$I-NGF to human melanoma cell membranes in the presence of WGA. In order to inhibit binding, these authors added WGA 30 min before $^{125}$I-NGF, whereas
WGA added after the binding of $^{125}$I-NGF had equilibrated did not affect the total amount of $^{125}$I-NGF specifically bound. An increase in the human placental IR affinity was reported after elution from lectin columns, which was speculated to be a consequence of the removal of a putative IR inhibitor during lectin affinity chromatography.

In contrast to IGF1R and IR, the binding of $^{125}$I-IGF-II to its receptor(s) was generally not susceptible to lectin inhibition and/or enhancement under the employed experimental conditions. This finding strongly suggests differences in glycosylation between the high-affinity receptor(s) for IGF-II and the receptors that bind IGF-I and insulin from human placental membranes. $^{125}$I-labelled IGF-II was reported to bind to three types of receptors in human placental membranes: IGF1R, IGF2R and IR. Besides IGF1R and IR, a significant quantity of immunoreactive IGF2R was detected in the soluble placental membrane preparations using western immunoblot. Despite this, the results of other experiments strongly suggested preferential binding of $^{125}$I-IGF-II to the IR. Unlabelled IGF-II was a potent competitor of tracer levels of $^{125}$I-insulin binding to the IR from solubilised placental cell membranes. The receptor population eluted from an insulin–agarose column bound $^{125}$I-IGF-II and $^{125}$I-insulin equally well (unpublished results). The tracer concentrations of $^{125}$I-IGF-II, which were used in all our experiments, allowed it to bind preferably to the receptor with the highest affinity for this ligand. The candidate receptor for this role is IR-A, which binds IGF-II with a $K_d$ of 0.9 nM, whereas the $K_d$ for the IGF-II-IGF2R interaction is approximately 1.3 nM. Taken together with the literature data, the present findings might imply that the IR population from human placenta which binds $^{125}$I-IGF-II and the IR population which binds $^{125}$I-insulin are differently glycosylated. It was reported that the glycosylation state of the IR alters insulin and IGF-II binding to this receptor, which is in accordance with the present data. Although all cell types express both isoforms of the IR to various degrees (placenta expresses equal levels of the two IR isoforms), little is known about the mechanisms that underlie IR isoform-specific signalling.

The potential differences in glycosylation between IR-A and IR-B might imply different interactions of the two isoforms with different placental proteins, which could in turn result in different effector pathways. $N$-Glycans attached to IR-A and IR-B could be the signal for targeting the two isoforms to different membrane compartments, which is in accordance with the finding that IR-A and IR-B localise to different regions of the plasma membrane.

Many authors have suggested that the sugar moieties covalently attached to receptors play no significant role in ligand binding, but instead function to direct the transportation of the $de$ $novo$ synthesised receptors to the cell surface. In contrast, the requirement of carbohydrate moieties for high affinity binding of somatostatin, cholecystokinin and VIP receptors was observed as an inherent property of these receptors.
CONCLUSIONS

In summary, several aspects of this study are noteworthy. First, lectins modulate the binding of IGF-I and insulin to their receptors, but not the interactions of IGF-II with its receptor(s). Second, different lectins have significantly different effects on the ligand binding to human placental IGF1R and IRs, which is in accordance with differences in the glycosylation of the receptors. Moreover, the sequence of interactions (ligand-first to lectin-first) has great influence on the outcome of the binding reaction (enhancement or inhibition). Finally, the obtained results suggest that IR-A and IR-B from human placenta might be differently glycosylated.

Acknowledgement. The authors are grateful to the Ministry of Science of the Republic of Serbia for financial support (Project No. 143019).

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