Measuring the kinetic interaction of insulin analogs with solubilized full-length insulin receptors

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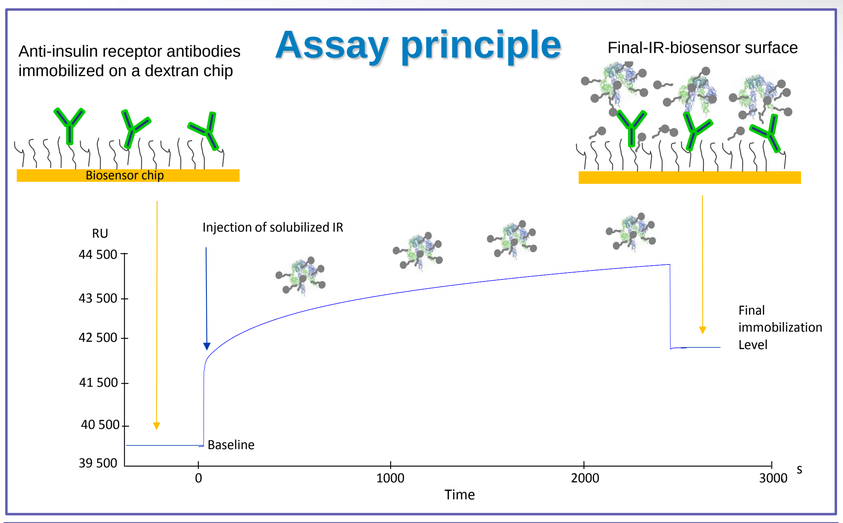
Assay principle



Introduction

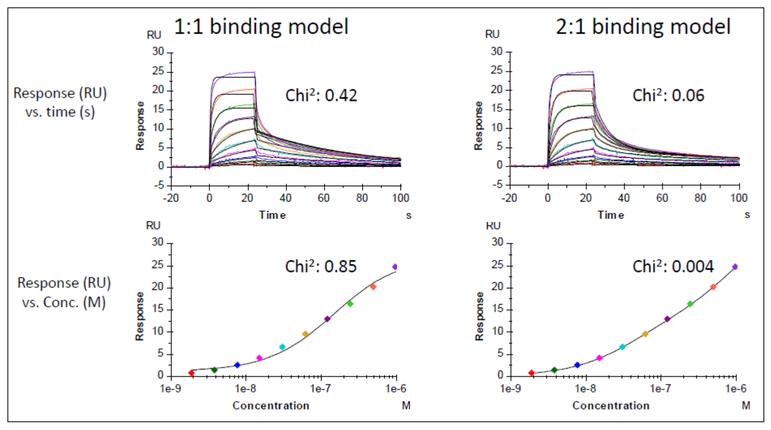
Considerable effort has gone into understanding the kinetics of interaction between insulin to its two binding sites on the insulin receptor (IR). A surface plasmon resonance (SPR) assay has been used to measure the interaction of human insulin (HI) with recombinant IR ectodomains. Here, these efforts were extended to an assay based on solubilized full-length IR isoform-A (IR-A) and –B (IR-B).

The study objective was to provide comparative receptor binding data for both IR-A and IR-B, including on- and off-kinetic rate constants, for different insulin analogs and insulin batches. Membrane preparations of cells overexpressing IR-A or IR-B were freshly solubilized using 1% n-Dodecyl-β-D-maltoside. A monoclonal IR-specific antibody was then immobilized to SPR sensor surfaces to capture solubilized IR. Functionality of the IR-A and IR-B surfaces was validated using 0.002−10 µM human insulin or insulin lispro, with each showing concentration-dependent saturation binding. Kinetic profile for the insulins indicated two separate binding sites on IR, one high affinity and one low affinity.

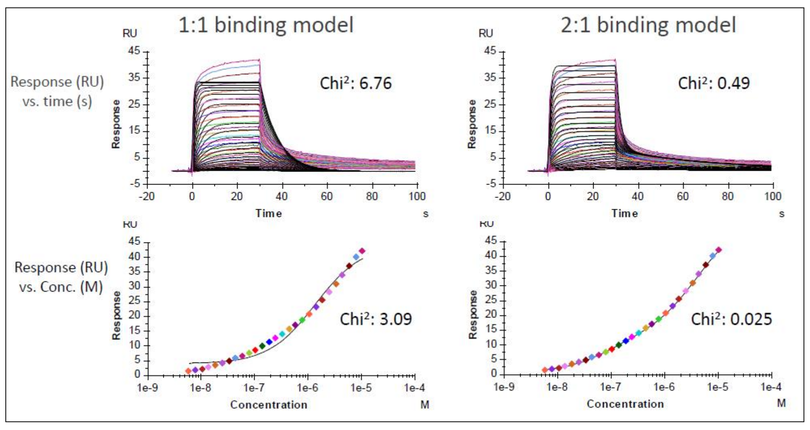
Insulin receptor isoform-A (IR-A)						
	k_{a1} (1/Ms)	k_{d1} (1/s)	K_{D1} (nM)	k_{a2} (1/Ms)	k_{d2} (1/s)	K_{D2} (nM)
Human insulin	2.7 ± 0.5*10^6	0.017 ± 0.003	6.3 ± 1.4	6.3 ± 0.8*10^5	0.14 ± 0.03	233 ± 50
Insulin Lis-Pro	3.4 ± 0.8*10^6	0.019 ± 0.004	5.8 ± 1.3	8.5 ± 1.0*10^5	0.17 ± 0.04	198 ± 51
Insulin receptor isoform-B (IR-B)						
	k_{a1} (1/Ms)	k_{d1} (1/s)	K_{D1} (nM)	k_{a2} (1/Ms)	k_{d2} (1/s)	K_{D2} (nM)
Human insulin	1.9 ± 0.3*10^6	0.013 ± 0.001	7.4 ± 1.3	4.1 ± 0.6*10^5	0.12 ± 0.02	293 ± 84
Insulin Lis-Pro	2.1 ± 0.3*10^6	0.016 ± 0.001	7.6 ± 1.1	5.9 ± 1.0*10^5	0.14 ± 0.02	251 ± 56

Comparison of interaction models

Human insulin interaction with IR-A

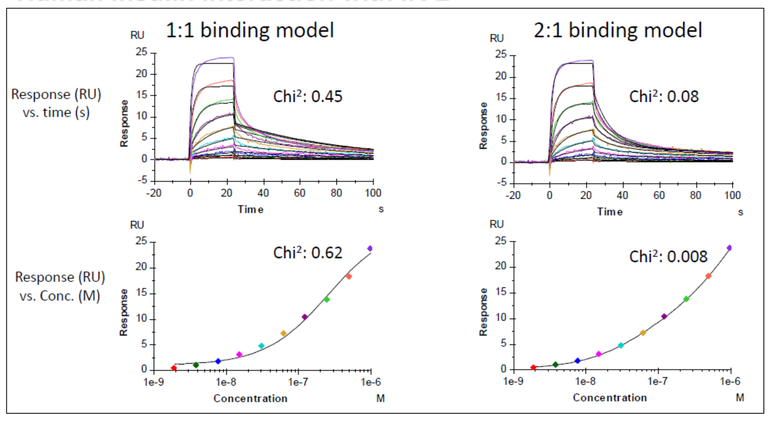


Insulin Lis-Pro interaction with IR-A

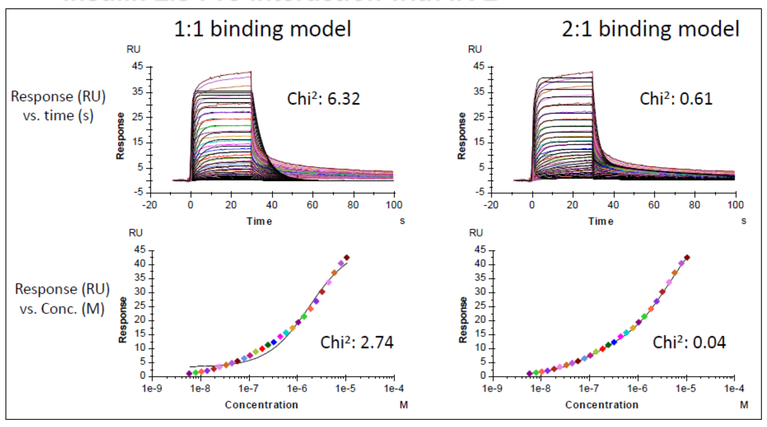


Sensorgrams and concentration-response plots for 10 concentrations (1.95 – 1000 nM) of human insulin and 27 concentrations (6 – 10000 nM) of insulin Lis-Pro

Human insulin interaction with IR-B

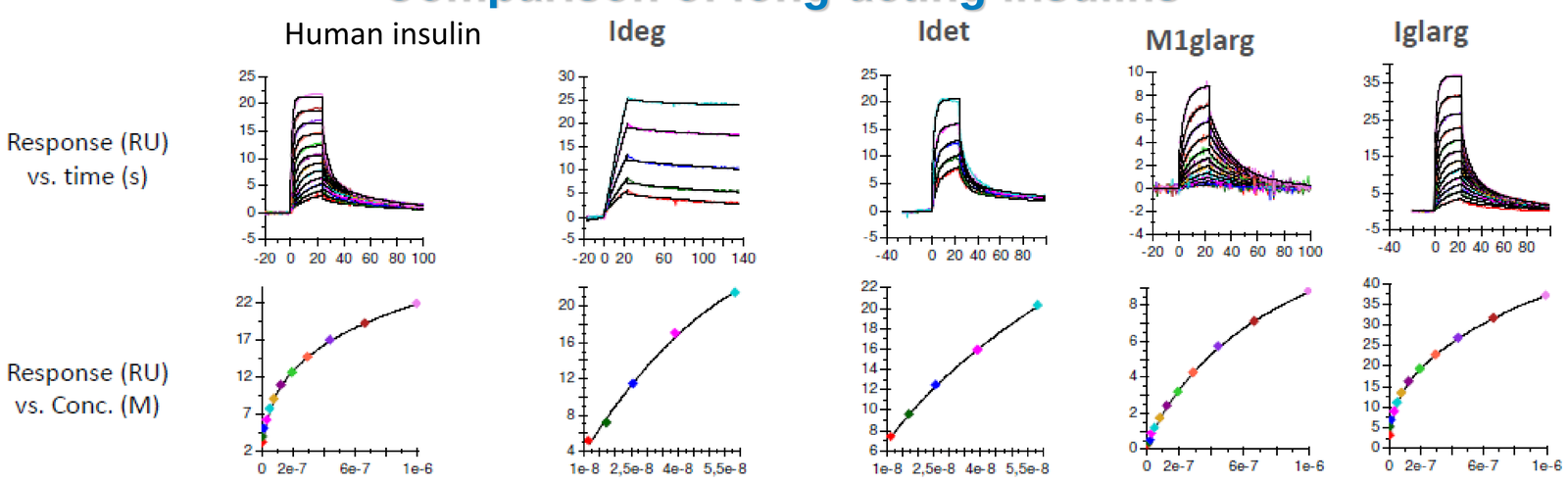


Insulin Lis-Pro interaction with IR-B



Sensorgrams and concentration-response plots for 10 concentrations (1.95 – 1000 nM) of human insulin and 27 concentrations (6 – 10000 nM) of insulin Lis-Pro

Comparison of long-acting insulins



- A model with two-binding sites provided the best fit for human insulin (PDB00178-6), insulin detemir (Idet), insulin glargine (Iglarg), the M1-metabolite of insulin glargine (M1glarg).
- Insulin detemir displayed an over-stochiometric interaction in our model, with binding exceeding the theoretical maximum. Further mechanistic studies may be needed to see whether additional ligand oligomerisation may explain this behavior.
- For insulin degludec (Ideg), a model with a 1:1 binding site gave the best fit with slow on- and off-rates. Unspecific binding of Ideg to a reference surface with immobilized IR-antibody only was observed. Experimental conditions needs to be optimized for this insulin.

Materials and Methods

The interaction experiments were performed using a Biacore™ T200 instrument and CM3 biosensor chips (GE Healthcare/Biacore, Uppsala, Sweden). Unless otherwise stated, the details below refer to optimized assay conditions, although numerous other conditions also have been tested during assay development.

Materials

- Membrane preparations of MEF cells overexpressing either full-length IR-A (UniProt: P06213-1), or full-length IR-B (UniProt: P06213-1)
- Reference Insulins: Insulin lispro (Humalog™ , batch no. C079720), or human insulin (Sanofi batch PDB 00178-6)
- Antibody for capture: Anti-insulin receptor alpha antibody (AbCam, Cat. No. ab36550, Lot No: GR170844, GR221398)

Insulin receptor solubilization

Aliquots of the membrane preparations (1 mg/mL protein) were thawed and centrifuged at 17,000×g for 1 min. The supernatant was removed and the membrane pellet was dissolved in 0.3 mL solubilization buffer [20 mM Hepes-NaOH (pH 7.8), 100 mM NaCl, 10 mM $MgCl_2$, 1% (w/v) n-Dodecyl-ß-D-maltoside and freshly added complete™ protease inhibitor tablets (Roche)] on ice for 60 min with occasional vortexing. The samples with solubilized membranes were then centrifuged at 17,000×g for 1 min. The supernatant was used for further experiments.

Immobilization of solubilized insulin receptor to CM3 chips

The anti-insulin receptor alpha antibody was covalently immobilized on CM3 chips using standard amine coupling (GE Healthcare/Biacore, Uppsala, Sweden). Solubilized IR was injected for 40 min over the antibody-coated surfaces.

Interaction experiments

All quality control experiments were performed using a Biacore T200™ instrument thermostated at 25°C. The characterization of compound interactions were conducted in a 10 mM Hepes buffer (pH 7.8) containing 150 mM NaCl, 0.05% Tween-20 and 10 mM $MgCl_2$. Concentration series of insulin analogues and the reference insulins were prepared in buffer and injected for 25 sec at a flow rate of 30 µL/min. The reference insulins were also injected as the first and last compound (at 100 nM) in order to monitor surface stability over time.

Data analysis

Raw data sensorgrams were double referenced by subtraction of curves from the reference surface and blank injections prior to data analysis, using Biacore™ T200 evaluation software 3.0. Data analysis was performed by global regression analysis of whole sets of sensorgrams from a concentration series. Average values and standard deviations were calculated using Excel and determined on the basis of at least three experimental series.

Summary & Conclusion

- A kinetic model accounting for two binding sites results in a best fit for human insulin or insulin Lis-Pro binding to both insulin receptor isoforms
- As long acting analogs, we tested the insulin glargine (Iglarg) and its major metabolite (M1glarg), insulin detemir and degludec
- Iglarg and M1glarg followed a 2:1 interaction model comparable to human insulin.
- Insulin detemir displayed an over-stoichiometric interaction, i.e. binding exceeded the theoretical maximum.
- Insulin degludec best fit a 1:1 binding model with slow on- and off-rates

In summary, we have developed a novel SPR biosensor-based assay, which allows the kinetic measurement of insulin to its two binding sites on the full-length insulin receptor. The methodology may be useful for the characterization of the different binding properties of insulin analogs and other ligand mimetic.

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