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A novel high-affinity peptide antagonist to the insulin receptor

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ABSTRACT

In this publication we describe a peptide insulin receptor antagonist, S661, which is a single chain peptide of 43 amino acids. The affinity of S661 for the insulin receptor is comparable to that of insulin and the selectivity for the insulin receptor versus the IGF-1 receptor is higher than that of insulin itself. S661 is also an antagonist of the insulin receptor of other species such as pig and rat, and it also has considerable affinity for hybrid insulin/IGF-1 receptors. S661 completely inhibits insulin action, both in cellular assays and in vivo in rats. A biosynthetic version called S961 which is identical to S661 except for being a C-terminal acid seems to have properties indistinguishable from those of S661. These antagonists provide a useful research tool for unraveling biochemical mechanisms involving the insulin receptor and could form the basis for treatment of hypoglycemic conditions.

Antagonists exist for most peptide hormone receptors, often truncated or otherwise modified analogs of the peptide hormone itself. One exception has been the insulin receptor to which no such antagonists derived from insulin have ever been identified, despite the fact that hundreds of insulin analogs have been described. Thus, so far the only available insulin antagonists have been insulin receptor antibodies [1], and due to the dimeric nature of antibodies their antagonistic properties sometimes depend on the context that they are used in.

In previous publications, we have described the identification of peptides that bind to two different sites on the insulin receptor and shown that heterodimers of two such peptides can be either agonists or antagonists depending on the orientation of the peptides [2,3]. In the present work, we describe the optimization of one such antagonist into an insulin receptor antagonist with high affinity and selectivity for the insulin receptor. Such an insulin receptor antagonist can be used as a biochemical research tool, and could also provide the basis for medical treatment of conditions such as hyperinsulinaemia caused by insulinoma as well as neonatal hyperinsulinaemic hypoglycemia.

Materials and methods

Peptide synthesis. S661 was synthesized by Fmoc chemistry on a Tentagel S RAM resin with a loading of about 0.25 mmol/g using automated, microwave-assisted synthesis on a Liberty Peptide Synthesizer (CEM Corp., Matthews, NC, USA). The couplings were performed with diisopropylcarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOAt) in *N*-methylpyrrolidone (NMP) for 5 min at up to 70 °C. Deprotection was performed with 20% piperidine in NMP for 3 min at up to 75 °C. After completion of synthesis the peptide was cleaved with trifluoroacetic acid/triisopropylsilane/water (92.5/5/2.5) for 2 h and precipitated with an excess of diethylether. The peptide was dissolved in 0.1 M Tris (pH 7) at about 5 mg/ml and 1.1 equivalent of [Pt(IV)ethylenediamine$_2$Cl$_2$]Cl$_2$ was added to oxidize the two cysteines to form an intramolecular disulfide bond [4]. After at least 1 h the solution was purified by standard RP-HPLC on a C18 column with TFA/acetonitrile. The fractions containing S661 were combined and lyophilized.

S961 was expressed in *Escherichia coli* as a fusion protein with an affinity tag. Further details will be published elsewhere (Shaw, unpublished results).

Receptor binding assays. Human insulin receptor (A and B isoform) was partially purified from transfected baby hamster kidney cells by wheat germ agglutinin (WGA) chromatography. Hybrids of insulin receptors and IGF-1 receptors were prepared as described elsewhere. Rat insulin receptor cDNA was obtained from Barry J. Goldstein, Jefferson University, and WGA-purified rat and pig insulin receptors were kindly supplied by Asser S. Andersen, Novo Nordisk. Two competition assays were used, a SPA (scintillation proximity assay) assay in microplates and a classical PEG precipitation assay in glass tubes. To analyze binding to the human insulin receptor SPA PVT Anti-mouse beads (Amersham Biosciences, UK) were incubated with IR antibody 83-7 [1] and insulin receptor for 5 h at room temperature. The SPA beads were washed twice with buffer ^{125}I-insulin (from Novo Nordisk A/S) was added. A two-fold dilution series of human insulin, S661 or S961 was prepared in

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a Packard Optiplate 96 and the SPA beads added. The final concentration of ^{125}I-insulin was 5000 cpm/200 μl and the buffer composition was 100 mM Hepes, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, 0.025% Tween 20. The plate was rocked gently for 18 h at room temperature, centrifuged and counted in a Topcounter. IC_{50} values were calculated by non-linear regression analysis. The same procedure was used for measurement of the affinity to hybrid receptors except that the receptor preparation used was derived from BHK-cells overexpressing both insulin receptors [5] and IGF-1 receptors and the tracer used was ^{125}I-IGF-1. The same procedure was also used to measure the affinity to IGF-1 receptors except that the receptor preparation was from BHK-cells overexpressing IGF-1 receptors, the antibody was IGF-1R antibody 24-31 [6] and the tracer was ^{125}I-IGF-1.

For measurement of the affinity to pig and rat insulin receptors a PEG binding assay was used where receptor was mixed with a

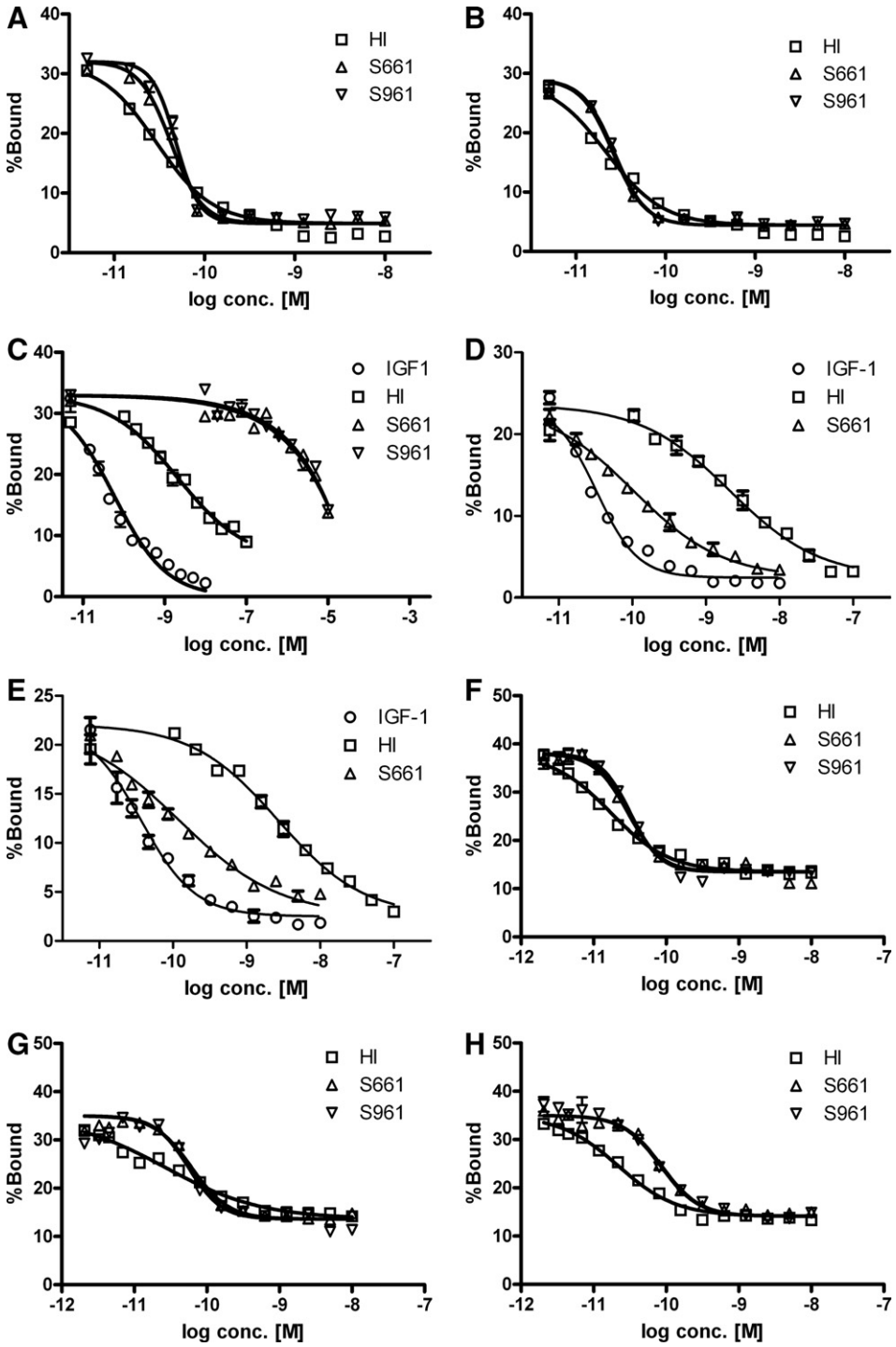

Fig. 1. All competition curves described under Results and discussion. The corresponding IC_{50} values are listed in Table 1. (A) SPA competition assay of ^{125}I-HI from HIR A. (B) SPA competition assay of ^{125}I-HI from HIR B. (C) SPA competition assay of ^{125}I-IGF-1 from IGF-1 receptor. (D) SPA competition assay of ^{125}I-IGF-1 from type A hybrid receptors. (E) SPA competition assay of ^{125}I-IGF-1 from type B hybrid receptors. (F) PEG competition assay of ^{125}I-HI from HIR B. (G) PEG competition assay of ^{125}I-HI from rat insulin receptor type B. (H) PEG competition assay of ^{125}I-HI from pig insulin receptor type B.

dilution series of peptide in a total volume of 200 µl of 100 mM Hepes, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.025% Triton X-100, 0.5% (w/v) HSA, 0.2% gammaglobulin and 2500 cpm ^{125}I-insulin and incubated for 18 h at room temperature. Receptor bound tracer was precipitated with 400 µl of 25% (w/v) PEG 8000 and the precipitate was washed once with 400 µl of 15% (w/v) PEG 8000 and counted on a Packard gamma counter. IC_{50} values were calculated using non-linear regression.

Inhibition of lipogenesis in primary rat adipocytes. Lipogenesis was determined using isolated primary rat adipocytes as described with minor modifications [2]. In brief, male rats at 80–100 g were killed, and the epididymal fat pads were removed and placed in a degradation buffer containing collagenase at 36 °C for 1–1.5 h under vigorous shaking. The cell suspension was filtered, and adipocytes were washed twice and resuspended in an incubation buffer (Krebs buffer, 1% HSA) and 100-µl aliquots were pipetted into 96-well Picoplates (Packard). Ten microliters of insulin and 10 µl of antagonist were added, and the assay was initiated by the addition of 10 µl of D-[3-^{3}H]glucose and glucose to a final concentration of 0.5 mM. The plates were shaken for 2 h, and the assay was stopped by the addition of MicroScint-E (Packard). Plates were counted in a TopCounter (Packard). All cells were incubated in the presence of 0.4 nM insulin (~90% of maximal insulin response) in the presence of increasing concentrations of S661/S961 (0–125 nM). Quadruplicate samples were run for every experiment. Data were normalized to the response without antagonists and the IC_{50} calculated.

Effect on plasma glucose in Zucker Obese rats. Male Zucker Obese rats, 16 weeks old (Charles River, USA) were fed ad libitum (Purina 5008 PMI, Nutrition International, USA) and fasted from 3 pm the day before experiment. Next morning, i.v. administration of S661/vehicle (1 ml/kg) with a polypropylene tuberculin syringe (30G needle) was followed 10 min later by i.p. administration of human insulin/vehicle ($t = 0$). Samples for measurement of blood glucose (full blood) concentration (BIOSEN 5040, Germany) were taken at −30, −20, 10, 20, 30, 40, 60, 80, 120, 180 min.

In the second experiment, the time between injection of S661/vehicle and insulin/vehicle was either 360 min or 10 min.

Results and discussion

Previously, we have described the identification of an insulin receptor antagonist called RB537 with the peptide sequence MAD YKDDDDKGSLDESFYDWFERQLGGGSGGSWLDQEWAWVQCEVYGR GCPSAAAGAPVPYPDPLEPRPG [2]. This sequence was minimized by removal of the affinity tags at each end and the sequence was further affinity optimized by changing a number of amino acids in the C-terminal region of the peptide. The peptide with the highest affinity obtained by this method was called S661 and had the peptide sequence GSLDESFYDWFERQLGGGSGGSSLEEEWAQIQCEVWG RGCPSY with the two cysteines connected by a disulfide bond and the C-terminus being an amide. This peptide was synthesized by microwave-assisted solid-phase peptide synthesis using the Fmoc strategy. The same peptide sequence was also prepared by a biosynthetic method in *E. coli* (details to be published elsewhere), and in this case the C-terminus was an acid and the peptide was called S961. In a scintillation proximity assay (SPA) using ^{125}I-human insulin as tracer, the apparent affinity of S661 and S961 for both isoforms of the insulin receptor (HIR A and HIR B) was close to that of insulin (Fig. 1A and B). The competition curve was slightly steeper than that of insulin, and we also found that steady-state conditions were difficult to achieve in the case of the peptides. Thus, the reported IC_{50} values are the ones obtained under the standard conditions used in the assay of insulin analogs. The mechanistic aspects of the binding properties of S661 will be described in more detail elsewhere. In a similar competition assay using the IGF-1 receptor with ^{125}I-IGF-1 as the tracer, the IC_{50} value was about 1 µM for both S661 and S961 (Fig. 1C). Thus, the selectivity of the peptides for the insulin receptor versus the IGF-1 receptor is more than 4 orders of magnitude which is significantly greater than the 30- to 1000-fold selectivity exhibited by insulin and IGF-1 for their cognate receptors. The explanation for the high selectivity is that the Site 1 peptide which makes up the N-terminal end of S661 binds to both the insulin receptor and the IGF-1 receptor with an affinity in the 0.1–1 µM range whereas the Site 2 peptide which makes up the C-terminal end has no measurable affinity for the IGF-1 receptor. Thus, the 4 orders of magnitude increase seen for insulin receptor affinity when the two peptides are joined together is not accompanied by an increase in IGF-1 receptor affinity. Another question pertaining to receptor selectivity is the affinity for hybrid receptors which are composed of one alpha-beta unit from the insulin receptor and one alpha-beta unit from the IGF-1 receptor and are present (presumably formed in a stochastic fashion) on all cells expressing both receptors. The hybrid receptor assay is a variation of the SPA assay wherein a HIR antibody is bound to the SPA beads and the receptors are WGA-purified receptors from BHK-cells overexpressing both HIR and IGF-1 receptors (thus presumably containing a high proportion of hybrid receptors). The SPA beads bind HIR and hybrids but IGF-1 receptor is removed by washing. The competition assay is then performed using ^{125}I-IGF-1 which only binds to the hybrids and not to the HIR. As shown in Fig. 1D and E the affinity of the peptides for the hybrid receptors was significantly higher than for IGF-1 receptors as would be expected for a receptor which has a functional insulin receptor Site 2 on one of the alpha subunits. Since hybrid receptors mainly signal as IGF-1 receptors, a high concentration of the peptides will also be expected to be able to antagonize IGF-1 action to some extent in cells with a high proportion of hybrid receptors. Finally, we addressed the question of species selectivity by measuring the affinity of S661 to the insulin receptor from pig and rat. Since the SPA binding assay used relies on a mouse antibody (Ab 83-7) which does not recognize the rat receptor we used a PEG precipitation assay for comparison of the rat and pig receptors (both isoform B) with HIR B (Fig. 1F–H). The affinity of S661 and S961 was slightly lower in the animal species as might be expected since all affinity maturation of the pep-

Table 1
Affinity of human insulin, IGF-1, S661, and S961 for various receptors

IC_{50} (mol/l)	HIR-A[a]	HIR-B[a]	HIR-B[b]	HIGF-1R[a]	A-hybrid[a]	B-hybrid[a]	Rat IR[b]	Pig IR[b]
Human Insulin	$2.9 * 10^{-11}$	$2.2 * 10^{-11}$	$1.7 * 10^{-11}$	$2.1 * 10^{-9}$	$2.1 * 10^{-9}$	$2.7 * 10^{-9}$	$2.7 * 10^{-11}$	$2.2 * 10^{-11}$
IGF-1	nd	nd	nd	$5.9 * 10^{-11}$	$3.3 * 10^{-11}$	$3.7 * 10^{-11}$	nd	nd
S661	$4.2 * 10^{-11}$	$2.6 * 10^{-11}$	$3.0 * 10^{-11}$	$1.3 * 10^{-6}$	$1.0 * 10^{-10}$	$1.2 * 10^{-10}$	$6.1 * 10^{-11}$	$8.6 * 10^{-11}$
S961	$4.8 * 10^{-11}$	$2.7 * 10^{-11}$	$3.2 * 10^{-11}$	$6.3 * 10^{-7}$	nd	nd	$5.6 * 10^{-11}$	$8.4 * 10^{-11}$

nd = not determined.
[a] SPA-assay.
[b] PEG-assay.

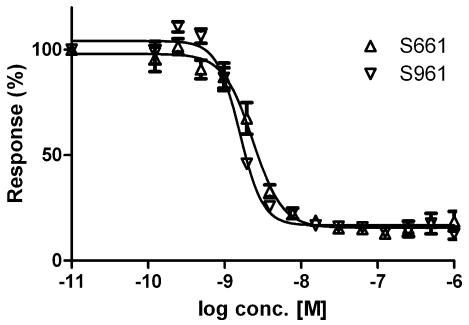

Fig. 2. Inhibition by S661 and S961 of insulin stimulated lipogenesis in primary rat adipocytes stimulated with 0.4 nM insulin (corresponding to ~90% of max response).

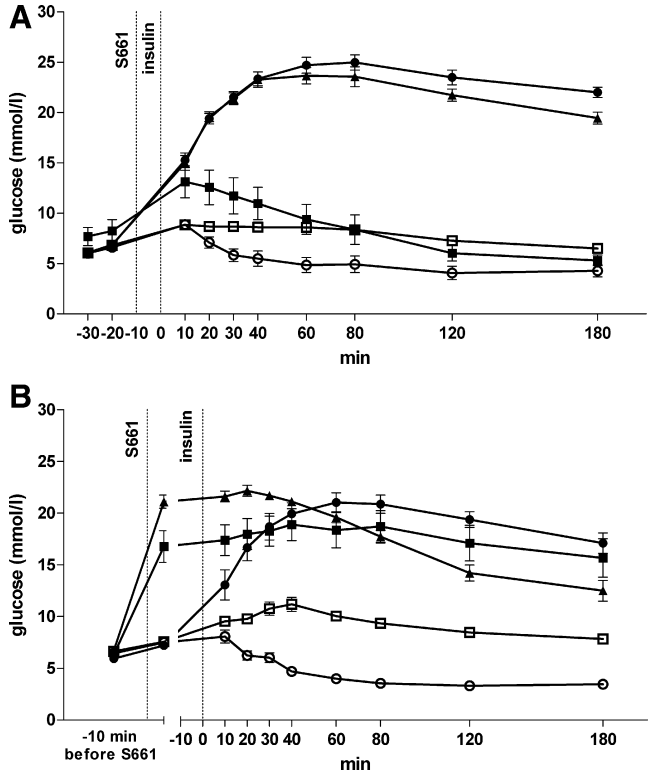

Fig. 3. (A) Blood glucose of male Zucker Obese rats after administration of S661 followed by insulin ($N = 5$ or 6 in each group, mean ± SEM). ■, 3 nmol/kg S661 at time −10 min and 30 nmol/kg human insulin at time 0. ▲, 30 nmol/kg S661 at time −10 min and 30 nmol/kg human insulin at time 0. ●, 100 nmol/kg S661 at time −10 min and 30 nmol/kg human insulin at time 0. ○, vehicle at time −10 min and 30 nmol/kg human insulin at time 0. □, vehicle at time −10 min and vehicle at time 0. (B) Blood glucose of male obese Zucker rats after administration of S661 followed by insulin ($N = 7$ in each group, mean ± SEM). ■, 30 nmol/kg S661 at time −360 min and vehicle at time 0. ▲, 30 nmol/kg S661 at time −360 min and 30 nmol/kg human insulin at time 0. ●, 30 nmol/kg S661 at time −10 min and 30 nmol/kg human insulin at time 0. ○, vehicle at time −10 min and 30 nmol/kg human insulin at time 0. □, vehicle at time −10 min and vehicle at time 0.

tide was performed on the human insulin receptor. The affinity decrease, however, was not big enough to cause concern about the possible utility of S661 and S961 as an antagonist of insulin receptors in those species. All IC_{50} values derived from the experiments shown in Fig. 1 are summarized in Table 1.

In order to confirm the antagonistic properties of S661 and S961 in whole cells we examined their effect in a lipogenesis experiment in primary rat adipocytes (Fig. 2). When increasing concentrations of S661 and S961 were added to adipocytes stimulated with 0.4 nM insulin (corresponding to about 90% of maximal stimulation), the peptides were able to inhibit the insulin stimulated lipogenesis response with an IC_{50} of 2.3 nM for S661 and 1.6 nM for S961.

To examine the in vivo effect of S661, we performed an intravenous bolus administration of S661 to male Zucker Obese rats, with or without a subsequent intraperitoneal injection of insulin. As shown in Fig. 3A, administration of S661 resulted in a marked increase in blood glucose levels. A dose of 3 nmol/kg was not enough to counteract the effect of a subsequent dose of 30 nmol/kg human insulin whereas both the 30 nmol/kg and the 100 nmol/kg doses were sufficient to block the effect of insulin. In order to estimate the duration of the antagonist action we administered S661 (30 nmol/kg) at different time points before the administration of insulin, and as shown in Fig. 3B the effect persisted for at least 6 h at the doses used. This is consistent with an estimated $t_{1/2}$ of about 90 min for ^{125}I-labelled S661 after intravenous bolus administration to male Sprague–Dawley rats (data not shown).

In conclusion, the insulin receptor antagonists S661 and S961 exhibit high affinity and selectivity for the insulin receptor and can be used to unravel the role of insulin receptor signaling in both in vivo and in vitro experiments. Also, they might provide the basis for novel treatments of disorders involving hypoglycemia induced by hyperinsulinaemia such as e.g. insulinoma.

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