Peptides 100 (2018) 18–23

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Controlled intramolecular antagonism as a regulator of insulin receptor maximal activity

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ARTICLE INFO

Keywords:
Controlled intramolecular
Antagonism
Regulator of insulin receptor maximal activity

ABSTRACT

In the treatment of insulin-dependent diabetes the risk of a fatal insulin overdose is a persistent fear to most patients. In order to potentially reduce the risk of overdose, we report the design, synthesis, and biochemical characterization of a set of insulin analogs designed to be fractionally reduced in maximal agonism at the insulin receptor isoforms. These analogs consist of native insulin that is site-specifically conjugated to a peptide-based insulin receptor antagonist. The structural refinement of the antagonist once conjugated to insulin provided a set of partial agonists exhibiting between 25 and 70% of the maximal agonism of native insulin at the two insulin receptor isoforms, with only slight differences in inherent potency. These rationally-designed partial agonists provide an approach to interrogate whether control of maximal activity can provide glycemic control with reduced hypoglycemic risk.

1. Introduction

Modern insulin regimens are an essential component of diabetes therapy, but seldom succeed in mimicking endogenous pancreatic secretion of the hormone. The application of integrated basal and bolus insulin therapy is the most effective way to safely control plasma glucose and minimize the risk of diabetic microvascular complications, such as retinopathy, neuropathy, and nephropathy [1]. Currently available "modern insulins" include the basal analogs detemir [2], glargine [3–5] and the recently introduced insulin analog degludec [6]. Bolus therapy is commonly provided by the insulin analogs lispro [7], aspart [8] and glulisine [9]. Despite unquestionable benefits, insulin therapy poses a danger to patients as a result of its narrow therapeutic index, and patients repeatedly identify the fear of overdose and the resultant hypoglycemia as the primary reason for intentional underdosing [10].

These concerns have promoted the search for an insulin with a broader therapeutic index, ideally one less efficacious during hypoglycemia but not lessened in its ability to normalize hyperglycemia. We questioned whether controlled adjustment of the maximal *in vitro* insulin activity might provide an approach to an insulin that was less prone to excessive action, but without compromising its inherent potency in correcting hyperglycemia. Currently, the relationship between maximal *in vitro* activity of an insulin analog and the ability to lower blood glucose is unknown. The maximal activity of native insulin is expected to occur at supraphysiological insulin concentrations, complicating the ability to assess the effect of maximal activity on glycemic control. Therefore, insulin analogs with a measured reduction in maximal activity can provide an opportunity to explore the relationship between maximal *in vitro* receptor activity and pharmacologic effects. In order to attenuate insulin's maximal activity, without destroying its inherent potency, we desired a creative means to alter its receptor interaction in a controlled fashion.

The insulin receptor (IR) is a constitutively dimerized tyrosine kinase which exists in two splice variants, IRA and IRB, which vary in relative tissue expression levels and envisioned physiological function [11]. Each monomeric domain of the receptor contains two insulin binding sites arranged in an anti-parallel configuration. Binding of insulin to receptor site 1 and 2 in the dimeric receptor cross-links the two monomeric domains, leaving two remaining unoccupied binding sites. As a result of this unique binding configuration, insulin displays negative cooperativity at its receptor [12,13]. At low insulin concentrations there is steadily increased activity until at a point of higher concentration decreased activity results from additional association with the previously unoccupied binding sites.

We aimed to exploit the negative cooperativity of the insulin

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https://doi.org/10.1016/j.peptides.2017.11.022
Received 15 October 2017; Received in revised form 28 November 2017; Accepted 30 November 2017

receptor to create a series of insulin analogs with high inherent potency, but reduced maximal activity relative to native insulin. It had been previously shown that covalently dimerized insulins have high potency at the insulin receptor, but variable degrees of maximal activity [14,15]. These dimers serve as a useful proof of concept, but are limited in value by the perceived synthetic complexity to adjust the magnitude of the effect. We aimed to expand on this dimer-approach by creating a series of peptides that differ in maximal activity but with minimal structural change. We envisioned the possibility that a covalent dimer of insulin with an insulin receptor antagonist might lessen maximal activity and the magnitude of the effect could be logically controlled through chemical refinement of the antagonist. Hypothetically, the presence of the antagonist might either broaden the inherent therapeutic index or create an analog more easily opposed by endogenous counter-regulatory hormones. Either way, such an insulin could potentially translate to a therapeutic of sizable importance.

There are no known naturally occurring insulin receptor antagonists, and unlike several other hormones, a truncated version of insulin does not antagonize insulin action [16]. The insulin receptor antagonists studied in this report are peptides that can be prepared by chemical and biosynthetic methods, and have been biologically well-characterized. Schäffer and associates used phage libraries to identify peptides that bind to each of the two insulin receptor binding sites, as well as peptides that bind to a non-classical binding site [17]. Peptides that consisted of a single binding motif bound to the insulin receptor with low affinity [17], while peptides with two binding motifs were of much higher affinity, and antagonized insulin action [18]. These peptide antagonists served as a starting point in our research as we defined the necessary structural characteristics required for partial antagonism as a covalent conjugate to insulin.

2. Results

In search of a potent peptide that could be suitably used as an intramolecular brake on insulin agonism, we first created a modified version of the previously reported insulin antagonist peptide S661[19] (Table 1). The original peptide contains two insulin receptor binding sites, one of which is located at the N-terminus of peptide **1** and a second site at the C-terminus directed to an independent site on the insulin receptor. They are connected by a flexible GlyGlySerGlyGlySer hexapeptide linker. The C-terminal peptide binding site contains a disulfide bond reported to enhance affinity for the insulin receptor [17–19]. We explored the necessity of a disulfide bond by mutating the two cysteines to serine, resulting in peptide **2**, (Table 2). Like S661, this peptide (**2**) did not display any agonism at either insulin receptor isoform. Furthermore, it fully antagonized native insulin with an IC_{50} of 19.1 and 14.9 nM at the IRA and IRB isoforms, respectively (Fig. 1A and B). To explore whether the two binding sites were operating independently of one another we synthesized an additional peptide (**3**) where the two binding sites are of inverted order (Table 1). The resulting peptide was similarly tested for agonism and antagonism at the two insulin receptor isoforms, as previously shown with peptide **2**. This peptide displayed a similar biochemical character which was devoid of agonism but fully capable of antagonizing insulin action. Its antagonism at the IRA and IRB receptor isoforms was determined to be similar to peptide **2** with measured IC_{50} values of 35.8 and 14.2 nM, respectively (Fig. 1A, B). This last observation stands in contrast to the agonism observed in peptides analogous to peptide 3 that include a single intramolecular disulfide [18,19]. Our results indicate that the two insulin receptor binding sites contained within S661 are interchangeable in their relative order and that the single disulfide could be eliminated.

Having eliminated the disulfide it was now possible to introduce single cysteine residues at either end of the antagonist peptides, without the possibility of disulfide formation. These individual cysteine residues could be used for purposes of chemical ligation to insulin. We investigated the ability of the N- and C-terminal cysteine peptide analogs of single insulin receptor binding sites to antagonize native insulin (peptides **4–7**), (Table 1). While only the results at the insulin receptor B isoform are shown, similar results were obtained at the insulin receptor A isoform in every instance. Unlike peptide **2** and **3,** none of these four peptides were able to antagonize native insulin action, regardless of where the terminal cysteine was positioned (Fig. 1C, D). These results confirm the prior report that both insulin receptor binding sites are required to achieve antagonism [19].

To enrich the pool of peptides that might be conjugated to insulin and more importantly the specific order in the two binding sites that the peptide might present to the native insulin structure we prepared and analyzed a number of biosynthetic insulin analogs, using a fully potent, two chain insulin receptor agonist [20]. In these analogs, the antagonist was positioned as a linear fusion at either the N-terminus of the A-Chain, or the C-terminus of the B-Chain. It was concluded from these studies that peptide **3,** when positioned as an extension to the C-terminus of the B-Chain, was the most effective antagonist. In this orientation, the presence of the peptide **3** sequence as a linear fusion to insulin completely suppressed the ability of the insulin analog to signal at the insulin receptors (*data not shown*)[20]. The selective proteolytic removal of peptide **3** as a covalent extension to the B-chain restored insulin activity [20]. This demonstrates that the absence of insulin agonism in the biosynthetic insulin-peptide **3** fusion analog was a function of the internal brake on activity imposed by the antagonist peptide **3** [20].

To build upon the findings established with biosynthetic insulin fusion analogs we explored the chemical assembly of insulin semisynthetic conjugates to the putative antagonists. The primary attraction of such analogs, relative to biosynthesis, is the ability to explore additional chemical diversity that is not possible to introduce otherwise. To simulate the linear extension that was achieved biosynthetically with peptide **3,** we focused our attention on the C-terminal lysine at B29 as the most appropriate location for chemical conjugation. This lysine residue was selectively modified using an N-hydroxysuccinimide (NHS) ester of S-trityl, 3-thiopropionate, in an aqueous buffer at a pH of 10 (Fig. 2). At this pH, only the LysB29 side chain amine reacts to yield an insulin analog selectively modified, without reaction at the two N-terminal amines. The B29-modified insulin displayed full insulin agonism and only slightly reduced potency at both receptor isoforms (*data not shown*) [20]. Removal of the S-trityl, protecting group was immediately followed by reaction with 2,2′-dithiobis(5-nitropyridine) (DNTP) to yield an insulin analog suitable for forming disulfide bonds

Table 1
Peptide-based antagonism of insulin action.

No.	Description	Peptide Sequence	IC_{50} IRA	IC_{50} IRB
1	**S661**	**GSLDESFYDWFERQLGGGSGGSSLEEEWAQIQCEVWGRGCPSY**	**2.4**	**7.4**
2	**Ser-analog No. 1**	**GSLDESFYDWFERQLGGGSGGSSLEEEWAQIQSEVWGRGSPSY**	**19.1**	**14.9**
3	**Inverse No. 2**	**SLEEEWAQIQSEVWGRGSPSYGGSGGSGSLDESFYDWFERQLG**	**35.8**	**14.2**
4	**N-Cys, site #1**	**CGSLDESFYDWFERQLG**	–	–
5	**C-Cys, site #1**	**GSLDESFYDWFERQLGC**	–	–
6	**N-Cys, site #2**	**CSLEEEWAQIQSEVWGRGSPSY**	–	–
7	**C-Cys, site #2**	**SLEEEWAQIQSEVWGRGSPSYC**	–	–