Role of the intra-A-chain disulfide bond of insulin-like peptide 3 in binding and activation of its receptor, RXFP2

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A B S T R A C T

INSL3 is a member of the insulin-IGF-relaxin superfamily and plays a key role in male fetal development and in adult germ cell maturation. It is the cognate ligand for RXFP2, a leucine-rich repeat containing G-protein coupled receptor. To date, and in contrast to our current knowledge of the key structural features that are required for the binding of INSL3 to RXFP2, comparatively little is known about the key residues that are required to elicit receptor activation and downstream cell signaling. Early evidence suggests that these are contained principally within the A-chain. To further explore this hypothesis, we have undertaken an examination of the functional role of the intra-A-chain disulfide bond. Using solid-phase peptide synthesis together with regioselective disulfide bond formation, two analogs of human INSL3 were prepared in which the intra-chain disulfide bond was replaced, one in which the corresponding Cys residues were substituted with the isosteric Ser and the other in which the Cys were removed altogether. Both of these peptides retained nearly full RXFP2 receptor binding but were devoid of cAMP activity (receptor activation), indicating that the intra-A-chain disulfide bond makes a significant contribution to the ability of INSL3 to act as an RXFP2 agonist. Replacement of the disulfide bond with a metabolically stable dicarba bond yielded two isomers of INSL3 that each exhibited bioactivity similar to native INSL3. This study highlights the critical structural role played by the intra-A-chain disulfide bond of INSL3 in mediating agonist actions through the RXFP2 receptor.

1. Introduction

Insulin-like peptide 3 (INSL3; also known as relaxin-like factor and Leydig cell insulin-like peptide) is primarily produced by pre-natal and postnatal Leydig cells of the testis and the thecal cells of the ovary [1,18]. It is involved in mediation of fetal gonad translocation to the inguinal canal during development [18]. More recent studies have uncovered prominent new roles for INSL3 including the modulation of both male and female germ cell function [18,10]. Thus, INSL3 agonists and antagonists may have considerable potential as specific drugs for novel contraceptive approaches or infertility treatments in both sexes.

INSL3 is a member of the insulin-IGF-relaxin superfamily, which includes insulin, IGF-1, IGF-2, relaxin-1, relaxin-2, relaxin-3, INSL3, INSL4, INSL5 and INSL6. All peptides within this family (except IGF-1) have a common two-chain structure, with two inter-chain and one intra-A-chain disulfide bond. Each member (with the exception of INSL4 which is seemingly unstructured) retains a characteristic tertiary structure characterized by two short α-helices within the termini of the A-chain separated by a turn, and a long central α-helix within the B-chain [18,19].

RXFP2, the receptor for INSL3, is a member of the leucine-rich repeat containing G-protein coupled receptor family [11]. It is closely related to the relaxin receptor, RXFP1. Our previous studies have determined that the primary binding site of INSL3 to RXFP2 is mediated via key residues within the B-chain, namely, Arg(B16) and Val(B19), with His(B12) and Arg(B20) playing a secondary role. Together, these amino acids combine with the previously identified critical residue, Trp(B27), to form the receptor binding surface [15].

In contrast, little is known about those residues within INSL3 that are required to elicit RXFP2 receptor activation and downstream cell signalling, i.e. to confer agonist properties. The preliminary evidence suggests that these are contained principally within the A-chain [3,4]. As the intra-chain disulfide bond of the A-chain is a common motif for all peptide members of the relaxin

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family, we undertook to determine the role it plays in RXFP2 receptor binding and activation. Synthetic human INSL3 analogs were prepared in which the intra-A-chain disulfide bond was either removed or replaced by a dicarba homolog.

2. Materials and methods

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and peptide synthesis reagents (HBTU, HOBt) were obtained from GL Biochem (Shanghai, China), and Novabiochem (Sydney, Australia) respectively. Fmoc-amino acid-preloaded PEG polystyrene resins were obtained from Applied Biosystems (Melbourne, Australia). All solvents and reagents used for solid-phase synthesis were of analytical quality and used without further purification.

2.2. Reversed-phase (RP) HPLC

The mobile phase in all RP-HPLC systems consisted of 0.1% trifluoroacetic acid (TFA) in water for solvent A and 0.1% TFA in acetonitrile for solvent B. RP-HPLC was performed on a Waters HPLC system consisting of two pumps (model 600A), a gradient programmer, an injector equipped with a 2 mL sample loop, and a UV monitor. Elution was at a flow rate of 1.5 mL/min for analytical runs, and Vydac RP-P columns (C18, 4.6 mm, 250 mm; C4, 4.6 mm, 250 mm) were chosen as the stationary phases. Separations were with linear gradients of acetonitrile over 30 min at a flow rate of 1.5 mL/min. For preparative purification, elution was at a flow rate of 10 mL/min on Vydac RP-P columns (C18, 10.2 mm, 250 mm; C4, 10.2 mm, 250 mm) as stationary phase. Fractions containing the target peptide were manually collected and lyophilized.

2.3. Solid-phase peptide synthesis

Both the A- and B-chains of the INS3L analogs were separately synthesized using continuous flow Fmoc-solid-phase methodology on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA, USA) together with the following side chain protecting groups: Arg, Pbf; Asn and Gln, Trt; Asp and Glu, O-But; His, Trt; Lys, Boc; Ser and Thr, tBu. Selective cysteine S-protection was also employed: Cys(A11, B10), acetalidomethyl; Cys(A24), tert-buty1; and Cys(B22), trityl. For the synthesis of the intra-A-chain dicarba INS3L analog, a pseudoproline dipeptide [Leu-Ser(qMe,MPre)] was incorporated within positions 12 and 13, and Fmoc-L-allylglycine (Agl) was used in positions 10 and 15 of the INS3L A-chain.

2.4. Microwave-accelerated ring closing metathesis

Tricyclohexylphosphine[1,3-bis(2,4,6-trimethylphenyl)-4,5-dihydro-imidazol-2-ylidene][benzylidene]ruthenium(II) dichloride (2nd generation Grubbs’ catalyst) was supplied by Aldrich (Sydney, Australia) and stored under nitrogen. DCM and a 0.4 M solution of LiCl in DMF were degassed with high purity argon prior to use. Microwave reactions were carried out on a CEM Discover™ System fitted with the Benchmate™ option. The instrument produces a continuous focused beam of microwave irradiation at a maximum power delivery selected by the user, which reaches and maintains a selected temperature. Reactions were performed in 10 mL high pressure quartz microwave vessels fitted with self-sealing Teflon septa as a pressure relief device. The vessels employed magnetic stirrer beads and the temperature of each reaction was monitored continuously with a non-contact infrared sensor located below the microwave cavity floor. Reaction times were measured from the time the microwave reached its maximum temperature until the reaction period had elapsed (cooling periods not inclusive).

2.5. Unsaturated dicarba INSL-3 A-chain

A microwave reactor vessel was loaded with resin-bound peptide (0.5 g, 0.1 mmol), DCM (10 mL), 0.4 M LiCl in DMF (200 µL) and 2nd generation Grubbs’ catalyst (17 mg, 20 µmol, 20 mol%) in an inert environment. The system was sealed and the reaction mixture irradiated with 40 W of microwave energy and stirred at 100 °C for 1 h. The reaction mixture was cooled to room temperature, filtered through a fritted syringe and the resin washed with DCM (7 mL, 3 × 1 min) and MeOH (7 mL, 3 × 1 min) then left to dry in vacuo for 1 h. Post-metathesis, a small aliquot of resin-bound peptide was subjected to Fmoc-deprotection in the presence of 20% piperidine in DMF (1 × 1 min, 2 × 10 min) and washed with DCM (5 × 1 min), DCM (3 × 1 min) and MeOH (3 × 1 min). After acid-mediated cleavage, RP-HPLC and mass spectral analysis showed the formation of the desired cyclic peptide as two isomers in an approximately 40:60 ratio and a cyclic product to linear starting material ratio of approximately 70:30 as assessed by total UV absorbance at 214 nm. The resin-bound peptide was again subjected to identical metathesis conditions for additional 1 h, however, RP-HPLC and mass spectral analysis (see below) showed that there was no improvement in the conversion from linear to cyclic form.

2.6. Cleavage and purification

The A-chain, after the on-resin RCM reaction, was treated with 20% piperidine/DMF to remove the N-terminal Fmoc group. Final cleavage of both the A- and B-chains from their solid supports and simultaneous side chain deprotection was achieved by a 1.5 h treatment with TFA (94%) in the presence of scavengers: anisole (3%), 3,6-dioxo-1,8-octanedithiol (DODT, 2%) and triisopropylsilane (TIPS, 1%). The resin was filtered, washed with additional TFA, and the combined filtrate evaporated under nitrogen flow. The peptide products were precipitated with ice-cold ether and collected by centrifugation, washed twice with diethyl ether, air-dried and purified by preparative RP-HPLC.

2.7. Conversion of Cys(tBu) (A-24) to the 2-pyridylsulfenyl derivative

A-chain (20 µmol) and 2,2-dipyridyl disulfide (280 µmol) were added to TFA (0.9 mL) in an ice bath. Thioanisole (0.1 mL) and ice-cold trifluoromethanesulfonic acid in TFA (1 mL; 1:4, v/v) were added and the mixture stirred for 30 min on ice. The peptide was then precipitated with ice-cold diethyl ether, and the pellet collected by centrifugation, washed 3 times with ice-cold diethyl ether, air-dried and subjected to RP-HPLC purification.

2.8. Combination of [Cys11(Acm), Cys24(Pyr)] A-chain INSL3 with [Cys10(Acm), Cys22(SH)] B-chain

A-chain peptide (2 µmol) was dissolved in 50 mM NH4HCO3 (2 mL) and added to B-chain (2 µmol) in H2O (2 mL). The mixture was stirred vigorously at room temperature and the reaction was monitored by analytical RP-HPLC. After 1 h, the reaction was terminated by addition of neat TFA, and the target product was isolated by preparative RP-HPLC.

2.9. Formation of second inter-molecular disulfide bond in INS3L analogs via iodination

The [Cys11(Acm)]A-chain/[Cys10(Acm)]B-chain analog (1 µmol) was dissolved in glacial acetic acid (7.2 mL) and to this was added...
2.8 ml of 20 mM iodine/acetic acid and 0.54 ml of 60 mM HCl. After 2 h, the reaction was stopped by addition of 90 ml ice-cold ether [22], further cooled on dry ice for 3 min, and the pellet was collected by centrifugation employing an anti-explosive centrifuge (Spintron, GT-175FR), and purified by RP-HPLC. The peptides were chemically characterized as outlined below.

2.10. Chemical characterization

The purity of each synthetic peptide was assessed by analytical RP-HPLC and MALDI-TOF mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptides were also quantitated by amino acid analysis of a 24 h vapour phase acid hydrolyzate followed by derivatization with AccuTag chemistry and resolution of the labeled residues using a Shimadzu µbore RP-HPLC system (Melbourne, Australia).

2.11. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed on a Jasco J-815 spectropolarimeter and a 0.1 mm path length cell. The peptide concentration was 0.1 mg/ml in 10 mM sodium phosphate buffer containing 137 mM NaCl, pH 7.4. The far UV circular dichroism (CD) spectra of peptides were acquired between the wavelengths of 195–260 nm at room temperature with the resolution of 0.1 nm and bandwidth of 0.1 nm. Spectra were taken in the 195–260 nm wavelength range. Mean residue ellipticity ([θ] MR) was expressed in degrees cm²/dmol. Secondary structure was determined by comparison with the CD spectra of control peptides with known conformations.

2.12. Ligand binding assays

Competition ligand binding assays using HEK-293T cells stably transfected with human RXFP2 and utilizing either ¹²⁵I-INSL3
[13] or europium labeled INSL3 [17] were performed as previously described. 125I-INSL3 was kindly provided by Prof Pierre DeMeyts (Hagedorn Research Institute, Denmark). Data are expressed as mean ± SEM of % specific binding of triplicate determinations made from at least three independent experiments. Data were analyzed using GraphPad PRISM (GraphPad Inc., San Diego, USA) and a non-linear regression one-site binding model was used to plot curves and calculate pK_i values. Final pooled pK_i data were analyzed using one-way ANOVA coupled with Bonferroni’s test for multiple group comparison.

2.13. Functional cAMP assays

RXFP2 receptor activation was assessed using HEK-293T cells co-expressing RXFP2 and a cAMP reporter gene as previously described [16]. All experiments were repeated at least three times with triplicate determinations within each assay. Results are plotted as mean ± SEM of percent normalized response compared to the maximum response to INSL3. A non-linear regression curve was used to plot curves and calculate pEC50 values using GraphPad PRISM.

3. Results

To determine the importance of the intra-A-chain disulfide bond of human INSL3 on the structure and activity, two synthetic analogs were prepared in which the disulfide bond was removed. In one analog, [A(Cys10,15 → Ser)]INSL3, the two Cys residues which form this bond were replaced with the isosteric Ser residue. The second analog, [A(ΔCys10,15)]INSL3, had the two Cys residues deleted altogether. The A-chains of the two analogs were each assembled using Fmoc-solid-phase synthesis [6,7] and, after cleavage from the resin
and simultaneous removal of the side chain protecting groups, the S-tert-butyl group on Cys(A24) was converted to S-pyridinyl by reaction with 2,2'-dipyridyldisulfide in TFMSA [12]. Combination of the resulting [Cys11(Acm), Cys24(S-pyridinyl)] A-chain with the corresponding synthetic [Cys10(Acm), Cys22(SH)] B-chain occurred by thiolyis led to [Cys11(Acm)] A-chain/[Cys10(Acm)] B-chain. This intermediate was then converted to the respective final products, [A(Cys10,15 \rightarrow Ser)] INSL3 and [A(ΔCys10,15)] INSL3, by formation of the second disulfide bond between A11 and B10 by iodolysis. Both peptides showed the expected chemical characteristics as determined by MALDI-TOF MS and RP-HPLC (data not shown) (Figs. 1–3).

A third analog of INSL3 was prepared in which the intra-molecular disulfide bond was replaced by the isosteric, non-reducible dicarba bond [2]. The A-chain was assembled by conventional solid-phase synthesis [21] in which the Cys10,15 residues were replaced with the allyglycine residue (Agl) and concluded with the retention of the N-terminal residue. To facilitate the subsequent ring closure metathesis, a Leu-Ser(δMe,Me(3)) pseudoproline dipeptide was incorporated in positions 12 and 13 [21]. The tendency for pseudoprolines to form a bend in the peptide backbone can aid the cyclization of linear peptides. The dicarba bond was formed by ring closing metathesis under microwave irradiation [9]. After removal of the N-terminal Fmoc group, the resulting peptide was cleaved from resin and analyzed by RP-HPLC. Two peaks were identified each with the expected calculated molecular mass as determined by MALDI-TOF MS. Although our detailed NMR spectroscopic study on the related synthetic human relaxin-3 hormone bearing an intra-A-chain dicarba bond was unable to definitively discern between the two isoforms, the weight of evidence strongly suggested that the RP-HPLC earlier-eluting isomer is the conformationally more compact cis form whereas the extended trans conformer is the later eluting isomer [8]. This conclusion is supported by RP-HPLC thermodynamic principles where the peptide in the later eluting peak must have a more extended hydrophobic surface which is what would be expected for a trans conformation. On this basis, we conclude that for the synthetic human INSL3 dicarba isomers, the early-eluting RP-HPLC peak (Fig. 4, peak A) corresponds to the cis form and the later eluting peak is the trans isomer (Fig. 4, peak B). The relative ratio of the two isoforms was 40:60 based on UV absorbance and, in addition, there was approximately 30% unreacted starting peptide (Fig. 4, peak C). Conditions were not identified for improving the extent of metathesis. For the two isolated peptides, the Cys6A24(Bu) was displaced with the S-pyridinyl moiety by reaction with 2,2'-dipyridyldisulfide in TFMSA [12]. Combination of the peptide with the B-chain occurred by thiolyis and led to the [Cys11(Acm)] A-chain/[Cys22(Acm)] B-chain, after which the third and final disulfide bond between A11 and B10 was formed by iodolysis. The overall yield of the two intra-A-chain dicarba INSL3 peptides was 12 and 13% respectively (relative to the starting B-chain peptide). Analytical RP-HPLC (Fig. 5) and MALDI-TOF MS (data not shown) confirmed the high purity of the peptides.

![Fig. 4. RP-HPLC profile of crude synthetic human INSL3 A-chain following RCM and cleavage from the solid support. Peak A: INSL3 A-chain dicarba isomer 1. Peak B: INSL3 A-chain dicarba isomer 2. Peak C: unreacted [Agl10,15] INSL3 A-chain.](image)

The secondary structure of each of the four synthetic INSL3 analogs was determined by CD spectroscopy (Fig. 6). In phosphate buffer, each peptide exhibited a significant degree of α-helicity. However, the two INSL3 analogs lacking the intra-A-chain disulfide bond each showed a blue shift which was indicative of altered structure which, in turn, likely reflects the importance of the disulfide bond in contributing to the characteristic insulin-like conformation. The two dicarba INSL3 isomers each showed similar CD spectra to native INSL3 which indicates a similar overall secondary structure. This is despite the differences in the covalent geometry between disulfide and dicarba bonds. The former bond, at 2.05 Å, is significantly longer than the equivalent carbon-carbon bonds in the dicarba bond (1.54 and 1.34 Å respectively for each of the unsat-

![Fig. 5. Analytical RP-HPLC profiles of the two INSL3 dicarba isomers. Upper panel: isomer 1; lower panel: isomer 2.](image)

![Fig. 6. Circular dichroism spectroscopy profiles of INSL3 analogs.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>LGR8</th>
<th>125I-INSL3 binding pKᵢ</th>
<th>cAMP activity pEC50</th>
</tr>
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<td>INSL3</td>
<td>8.53 ± 0.10 (3)</td>
<td>10.22 ± 0.12 (4)</td>
<td></td>
</tr>
<tr>
<td>Isomer 1</td>
<td>8.90 ± 0.21 (3)</td>
<td>10.45 ± 0.01 (3)</td>
<td></td>
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<tr>
<td>Isomer 2</td>
<td>9.05 ± 0.07 (3)</td>
<td>10.37 ± 0.22 (3)</td>
<td></td>
</tr>
<tr>
<td>Ligand</td>
<td>9.22 ± 0.07 (6)</td>
<td>10.52 ± 0.04 (3)</td>
<td></td>
</tr>
<tr>
<td>INSL3</td>
<td>8.59 ± 0.04 (3)</td>
<td>10.38 ± 0.21 (3)</td>
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<tr>
<td>INSL3 A(C-10/15-S)</td>
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<td>10.38 ± 0.21 (3)</td>
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<tr>
<td>INSL3 A(A10/15C)</td>
<td>8.32 ± 0.21 (3)</td>
<td>10.38 ± 0.21 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*Binding affinities (pKᵢ: n = 3–6) and efficacy in stimulating cAMP accumulation (pEC50: n = 3–4) of INSL3 analogs in LGR8 expressing cells.*
Fig. 7. (A) Competition binding studies of human INSL3 compared to INSL3 analogs in HEK-293T cells stably transfected with RXFP2 using europium-labeled INSL3 as the ligand. Data are expressed as % specific binding and are pooled data from three experiments performed in triplicate. (B) Ligand stimulated cAMP accumulation in RXFP2 expressing cells measured using a pCRE-β-galactosidase reporter gene system. Data are expressed as % maximum INSL3 response and are pooled data from three experiments performed in triplicate.

Each synthetic INSL3 analog was tested for its ability to compete with $^{125}$I-INSL3 or europium-INSL3 binding in HEK-293T cells that had been stably transfected with RXFP2, the receptor for INSL3. Additionally, the INSL3 analogs were tested for their ability to activate cAMP in RXFP2 transfected cells. INSL3 bound to RXFP2 with high affinity ($^{125}$I-INSL3, $pK_i = 8.53 \pm 0.1$; europium-INSL3, $9.22 \pm 0.07$) and caused cAMP accumulation with high potency ($pEC_{50} = 10.22 \pm 0.12$) (Table 1). Each synthetic INSL3 analog retained high RXFP2 binding affinity relative to native INSL3 consistent with our previous results which have demonstrated that the B-chain alone is necessary for binding affinity [5,20] (Figs. 7A and 8A). Whereas both dicarba INSL3 isomers retained full cAMP activity compared to native INSL3 consistent with our previous results using human relaxin-3 [8] (Fig. 8A), in contrast, the peptides lacking the intra-molecular disulfide bond were completely inactive highlighting the essential role of the A-chain in receptor activation (Fig. 8B).

4. Discussion

Disulfide bonds are common structural motifs in naturally occurring cyclic peptides. In some cases, they constitute part of a peptide binding domain or active site where they can undergo disulfide exchange or reduction to release metal-chelating thiol groups. These events can be important for the activity of peptides and proteins. In most peptides, however, the cystine bridge serves only a skeletal, structural role to maintain the secondary and tertiary fold of the native peptide. In our earlier solution NMR spectral study of human INSL3 [15], we observed broad resonances from many residues within the INSL3 framework supporting the existence of significant internal dynamic conformational changes in its molecular core. This observation is consistent with that observed for H3 relaxin [14]. The same residues were found to be broadened in both peptides although to a more severe degree in INSL3 with several amide protons being undetectable. The broadening appears to be mainly centered on the CysA10-CysA15 disulfide bond making it likely that a rearrangement of the disulfide bond is the primary cause of the broadening. This interesting finding has raised the question that possible disulfide rearrangement may occur in which thiol group(s) may be produced and which, themselves, be involved in activation of INSL3 receptor. However, as the isosteric dicarba bond cannot undergo either cleavage or rearrangement, our results clearly show that the A-chain intra-molecular disulfide bond serves a primary structural role only to present other key residues within the A-chain for presentation to the RXFP2 receptor for its activation. Our subsequent studies will now focus on the clear identification of these residues.

In conclusion, solid-phase synthesis was used to prepare four intra-A-chain disulfide analogs of human INSL3. Two analogs were devoid of the intra-chain disulfide bond and the two others INSL3 isomers possessing an intra-chain dicarba bridge. By employing microwave irradiation technology, synthesis of the dicarba bond was successfully achieved by ring closure metathesis under mild conditions. It was found that the two dicarba INSL3 isomers retained full RXFP2 cAMP activity indicating that this isostere is a useful replacement of the physiologically labile disulfide bond. Studies are underway to replace all three disulfides of the INSL3 with dicarba bonds for subsequent use as a pharmacological probe as well as for pre-clinical applications.

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