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Synthesis, and Functional Properties of a Modified Human Insulin A-Chain: Implication in a 'Mini-Insulin' Structure Determination

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Abstract—The design and total synthesis of a novel insulin A-chain mutant, analogue **3**, is reported. In this compound, the cysteines implied in the two insulin inter-chain disulfide bridges are replaced by two serines (residues Ser^{A7} and Ser^{A20}) and the intra-A-chain disulfide bridge (residues Cys^{A6} and Cys^{A11}) is conserved. This A-chain analogue (**3**) has been tested in three in vitro cell culture assays, using insulin as a reference. The data clearly showed that analogue **3** mimics insulin effects on DNA synthesis, glucose uptake and glycogen synthesis without loss of potency as compared to insulin. To our knowledge, these are the first results showing that an isolated insulin chain displays functional properties similar to those of insulin. The implication of these new findings in insulin structure–function relationships and in a 'mini-insulin' structure determination is discussed. © 2002 Published by Elsevier Science Ltd.

Introduction

Insulin is one of the most widely studied peptide hormones which, so far, takes a major part in the therapeutical treatment of diabetes. It is composed of two polypeptide chains,[1] the A-chain and the B-chain which are linked by two inter-molecular disulfide bridges between the residues Cys^{A7}-Cys^{B7} and Cys^{A20}-Cys^{B19}, whereas an intra-molecular disulfide bridge joins the residues Cys^{A6}-Cys^{A11}. Insulin induces its biological effects through interaction with a specific membrane receptor, a glycoprotein consisting of two α- and β-subunits linked together to form a hetero-tetramer. The α-subunit is entirely extra-cellular and contains the insulin binding site. The β-subunit presents an extracellular domain linked to the α-subunit by disulfide bonds, a transmembrane region, and a cytoplasmic region which contains a tyrosine kinase domain activated upon ligand binding.[2–4]

Recently, the three-dimensional (3-D) structure of the complex insulin-receptor, has been determined by electron cryomicroscopy and 3-D reconstruction.[5] In the absence of a crystallographic structure of this complex, such an approach has provided new insights in the mechanism of insulin interaction with its receptor. However, major knowledge on the functional surface of insulin was deduced in the past from insulin structure–function relationships, using insulin variants. Of particular interest is mini-proinsulin, an inactive insulin analogue that contains a peptide bond between the A- and B-chains.[6] Although the mini-proinsulin crystal structure is identical to that of native insulin, it displays no biological activity. Thus, it has been proposed that conformational changes in the insulin hormone are required when insulin binds to its receptor.[7] Moreover, it has been shown that the C-terminus of insulin B-chain as well as residues close to this region are crucial for the interaction of insulin with its receptor.[8] Finally, it has been suggested that the N-terminal domain of the A-chain is also important for receptor binding and that the native α-helix observed in this region, a site of mutation in *Diabetes mellitus*, provides a preformed determinant for receptor recognition.[9]

In order to better understand the mechanism of interaction between insulin and its receptor, we report, here, on the total synthesis and the functional properties of a novel A-chain mutant of human insulin. The biological

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activity of this analogue has been investigated, using conventional in vitro assays, and insulin as a control. Effects of the A-chain mutant on DNA synthesis by fibroblasts, glucose uptake by adipocytes and glycogen formation by hepatocellular carcinoma cells (HepG2) were determined.

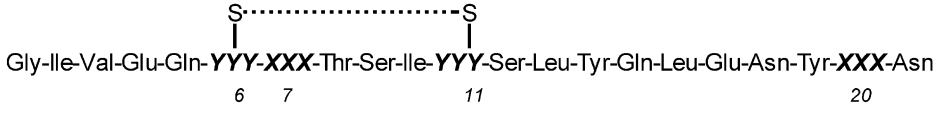
Results and Discussion

The human insulin A-chain analogue **3** presents the native intra-molecular disulfide bond between the residues Cys^{A6} and Cys^{A11} and two serines in positions 7 and 20 replace the cysteines implied in the two inter-chain disulfide bridge formed with the B-chain in the native insulin (Fig. 1). Indeed, cysteine thiol group is known to be unstable and spontaneously polymerizes under oxidative conditions. Therefore, several chemical groups such as *S*-thiomethyl, *S*-sulfonate, sulfonate and hydroxyl have often been used to mimic the thiol group in 3-D structures studies of insulin isolated chains.[10–13] From these studies, it has been recently deduced that the negative charge of the sulfonate groups exerts a destructuring effect, destabilizes the 3-D structure and affects the folding of an isolated insulin B-chain analogue. In contrast, the serine hydroxyl group promotes a greater 'insulin-like' conformation.[13] Based on these findings, Cys^{A7} and Cys^{A20} were therefore substituted by two serine residues in this study. Then, the effects of analogue **3** were determined in three biological assays. Indeed, insulin is known to display mitogenic properties on several cell types,[14] and to stimulate glucose uptake by several cell types, including hepatocytes and adipocytes,[15,16] with subsequent increased glycogen formation in hepatocytes.[16,17]

Peptide synthesis

The protected linear sequence corresponding to human insulin A-chain analogue **3** was assembled by solid-phase synthesis,[18] using Fast-Moc chemistry according to standard methods.[19] The acidolyzable protecting groups for the amino acid reactive side chains, *tert*-butyl (*t*Bu) for the residues Glu, Thr, Ser and Tyr and trityl (Trt) for the residues Cys, Asn and Gln, and the Super Acid Sensitive ResIN (SASRIN-TM®) were selected because a single acidolytic cleavage/deprotection step [using trifluoroacetic acid (TFA) in the presence of scavengers] leads to the bis(thiol) compound **2** (Fig. 1). Such procedure has been widely used by others and is known to provide good yields of the target peptide.[20–22]

The formation of the intra-molecular disulfide bridge is the following crucial step in the synthesis of analogue **3**. As disulfide bridges are present in many extra-cellular proteins, disulfide-containing peptides have long been an attractive challenge for chemical synthesis. Extensive works have been done in that domain and proteins bearing a single or multiple intra- and/or inter-chain disulfide bridges have been successfully synthesized.[20–22] It is generally accepted that native peptides can be correctly synthesized because their tertiary structure is largely determined by the amino acid sequence. Thus, the disulfide bridge does not bring structural information but simply plays a role in stabilizing the bio-active conformation.[21] To form the intra-molecular disulfide bridge of the analogue **3**, high dilution is recommended to obtain the disulfide bond in good yield and to prevent aggregation and disulfide scrambling. Several oxidation protocols to form disulfide bridges have been reported.[20,22] Oxidation by air in slightly alkaline conditions



(1) ***XXX*** = Cys(ox)[a] ***YYY*** = Cys(ox) — A-chain in native human insulin (The residues Cys 7 and Cys 20 are implied in the two interchain disulfide bridges with the B-chain while the residues Cys 6 and Cys 11 are linked to form the intra-chain disulfide bridge)

(2) ***XXX*** = Ser ***YYY*** = Cys(red)[b] — Insulin A-chain analog precursor bis(thiol)

(3) ***XXX*** = Ser ***YYY*** = Cys(ox) — Insulin A-chain analog

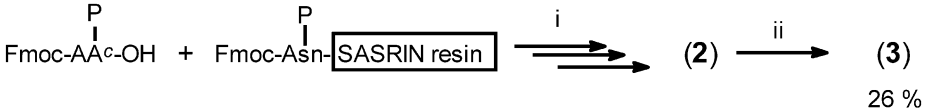


Figure 1. Synthesis of human insulin A-chain analogue **3**. [a]Cys(ox): cystine residue, [b]Cys(red): cysteine residue, [c]AA: amino acid, P: protective group for reactive side chains: *t*Bu (Glu, Thr, Ser and Tyr residues) and Trt (Cys, Asn and Gln residues). (i) FastMoc solid-phase synthesis: (a) *N*-Fmoc deprotection: piperidine in NMP; (b) amino acid activation: HBTU/HOBt/DMF/DIEA in NMP; (c) coupling: amino acid (10 equiv) in NMP; (d) resin cleavage and protective group removal: TFA/CH_2Cl_2/TIS/H_2O/1,2-ethanedithiol/thioanisole, 10:5:1:1:1:1, 4 h, rt. (ii) Disulfide bond formation by air oxidation: (a) 2 mM peptide concentration, 10 mM phosphate buffer pH 7.8, Et_3N (10 equiv), 48 h, rt; (b) semi-preparative RP-HPLC (100% H_20, 0.1% TFA→60% acetonitrile, 0.1% TFA, 3 mL/min, 40 min).

(10 mM phosphate buffer, pH = 7.8) has been chosen here as a simple and efficient method (Fig. 1). Triethylamine has been added, as previously reported.[23] After 48 h, the peptide **3** has been purified, lyophilized, and obtained with an overall yield of 26% starting from the resin.

In vitro assays of the analogue 3 biological activity

In order to determine whether analogue **3** was capable of inducing proliferative response, DNA synthesis was measured in 3T3-L1 fibroblasts.[24] Figure 2 shows the mitogenic effect of insulin. The hormone at 1 μM was found to increase [^{3}H]-thymidine incorporation by about 22%. The stimulation reached 45 and 75% at 5 and 10 μM, respectively. Thus, the results confirm the well known ability of insulin to stimulate DNA.[14] Analogue **3** was also found to stimulate DNA synthesis. The mitogenic activity of this peptide was dose-dependent: an increase of [^{3}H]-thymidine incorporation of 34, 40 and 50% was observed for 1, 5 and 10 μM, respectively (Fig. 2). The comparison of the mean values of incorporation reveals no significant differences between insulin and analogue **3** (Student's *t*-test, $p > 0.05$, data not shown). Thus, the effect of analogue **3** on [^{3}H]-thymidine labeling is similar to that of insulin.

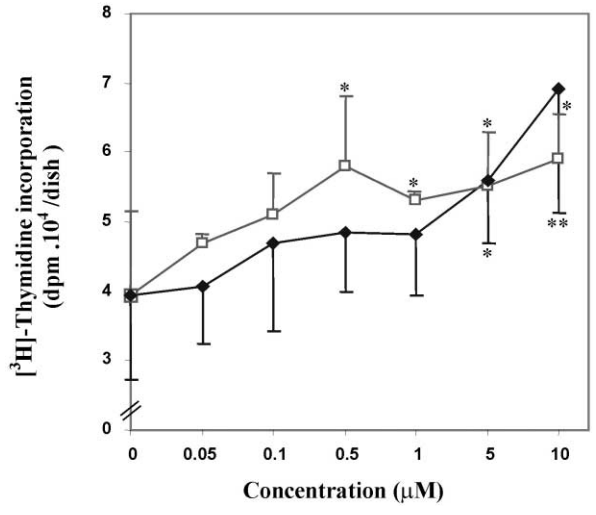


Figure 2. Effect of insulin and analogue **3** on [^{3}H]-thymidine incorporation. Cells were seeded at a density of 2×10^4 per dish in 10% FCS-containing DMEM. Insulin (◆) or analogue **3** (□) were added for 24 h at different concentrations. [^{3}H]-Thymidine (1 μCi/mL) was introduced for the last 4 h of incubation. Each point represents the mean ± SD of [^{3}H]-thymidine incorporation assays performed in quadruplicate (Student's *t*-test $*p < 0.05$, $**p < 0.01$).

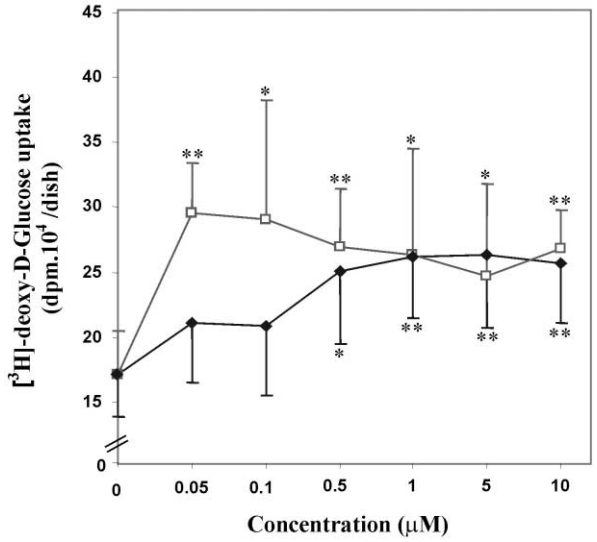


Figure 3. Effect of insulin and analogue **3** on glucose uptake in adipocytes. Increasing concentrations of insulin (◆) and analogue **3** (□) (0.05–10 μM concentrations) were added to the culture for 40 min. Each point represents the mean ± SD of glucose uptake values performed in triplicate (Student's *t*-test $*p < 0.05$, $**p < 0.01$).

Analogue **3** was also tested for its potential insulin-mimetic effect on the glucose uptake by adipocytes and glycogenesis by hepatocytes. First, the effect of increasing concentrations of insulin on [^{3}H]-2-deoxy-D-glucose (2DG) uptake on fully differentiated adipocytes were studied. As expected, glucose transport was increased by 45% after incubation with 0.5 μM insulin (Fig. 3). This effect reached a maximum (+ 50%) for 1 μM insulin. Similarly, analogue **3** was capable of enhancing glucose uptake. This effect was maximal (+ 50%) between 0.5 and 1 μM. Despite some experimental points corresponding to low concentrations suggest a higher activity of analogue **3**, the differences between insulin and analogue **3** values were not statistically significant (Student's *t*-test, $p > 0.05$, data not shown), indicating that insulin and analogue **3** effects on glucose uptake are highly similar.

Since, adipocytes are not considered as a major cell type for glucose conversion into glycogen, compared to hepatocytes, HepG2 cells were used to determine the effect of analogue **3** on glycogen synthesis. As expected, insulin produced a stimulation of + 35% at 0.05 μM and + 40% at 1 μM (Fig. 4). No significant effect was observed for higher concentrations. Incubation with analogue **3** also induced glycogenesis increase for the same concentration range as insulin. The effect was significant at 0.05 μM (+ 20%) and reached a maximum at 0.5 μM (+ 70%). From the statistical analysis, it was found that insulin and analogue **3** exert similar effects on glycogen synthesis.

Implication of these findings in insulin structure–function relationships

These results show that the insulin A-chain analogue **3** mimics three in vitro biological effects of insulin without major loss of potency as compared to insulin itself. Although other in vitro studies, including investigation on cell surface binding and signaling events, are required to better characterize the biological activity of the present molecule, our data show for the first time that an isolated insulin chain may have similar functional properties as insulin. So far, it was rather believed that both insulin A- and B-chains were needed to induce biological activities and that each chain contributed to the functional activity of insulin.[25,26] Most of the previous studies on insulin structure–function relationships

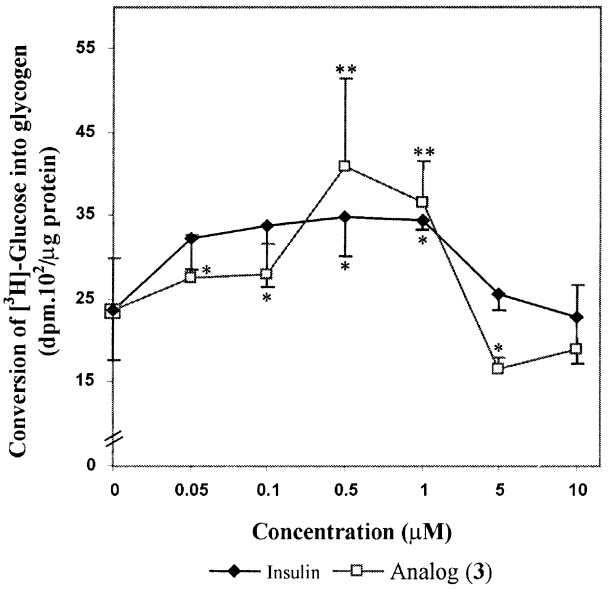


Figure 4. Effect of insulin and analogue **3** on glycogen synthesis in hepatocytes. Insulin (◆) and analogue **3** (□) were added to the cultures at increasing concentrations (0.05–10 μM concentration) for 4 h. Each point represents the mean ±SD of glycogen synthesis assays performed in triplicate (Student's *t*-test $*p<0.05$, $**p<0.01$).

have been done on insulin bearing punctual modifications. They have revealed that a number of amino acids are crucial at the binding surface, such as the residues Gly^{A1}-Gln^{A5}, Tyr^{A19} and Asn^{A21} belonging to the A-chain and Val^{B12}, Tyr^{B16}, Gly^{B23}-Tyr^{B26} present in the B-chain.[26]

Our study on the A-chain analogue **3** is in agreement with previous reports showing that the residues present in the A-chain play a crucial role in biological activity. In particular, the biological activity of an insulin variant in which the intra-A-chain disulfide bridge was suppressed, replacing the Cys^{A6} and Cys^{A11} residues by two serines, has been reported.[27] In the absence of this disulfide bridge, an important structure reorganization was observed in the A-chain N-terminal region, accompanied by a reduction of biological activity. Earlier, Baker et al. suggested that the N-terminal helix is a preformed element of crucial importance for the biological activity.[26] In contrast, Nakagama and Tager suggested that this helical structure may not be essential for receptor binding.[28] To get insights in this problem, one may first address the question of what is the role of the A-chain intra-molecular disulfide bridge in the insulin structure. Indeed, although its position in the A-chain sequence is approximately between the two A-chain helices, this intra-chain disulfide bridge might be responsible for the stability of the N-terminal one. The determination of the 3-D structure of analogue **3** may shed some light on this question. Preliminary data from a NMR study performed in our laboratory have recently shown that the structure of this mutant in solution significantly differs from that of the A-chain observed in the insulin molecule. In particular, the N- and C-terminal helices present in the native insulin A-chain could not be detected in our analogue **3**. However, the cyclic region located between the residues Cys^{A6} and Cys^{A11} was clearly characterized, as evidenced by several NOE cross peaks reflecting a structural fold of the concerned region. Further detailed NMR and modeling studies are in progress to better characterize the structure of our A-chain analogue. Moreover, the importance of other A-chain residues have been reported. In particular, it has been shown that both the aliphatic side chain at position A2 and A3,[28] and the Tyr^{A19} hydroxyl group are significant for binding to the receptor.[29,30]

On the other hand, our work raises another important question: if our modified A-chain analogue is sufficient to elicit 'insulin like' signal, what could be the role of the B-chain? Indeed, several studies have reported the contribution of some B-chain residues in the binding to the insulin receptor. In particular, the amino acids present in the β-turn domain (residues Gly^{B20}-Gly^{B23}) and aromatic residues of the vicinity were found to be essential.[8,31–34] As a hypothesis to explain our findings, we may suggest that these B-chain elements could not play a determinant role in the binding of insulin to its receptor. Indeed, the fact that a conformational change is required when insulin interacts with its receptor may complicate the interpretation of results derived from structure–function relationships. Finally, B-chain residues might only be useful to provide full biological activity to insulin, reinforcing the affinity of insulin for its receptor and/or stabilizing the conformation of the A-chain.

Implication of the data in a 'mini-insulin' structure determination

Another approach has been used by other groups, consisting in testing small peptides for their 'insulin-like' properties. Indeed, several insulin derived fragments were found to display residual bio-activity in vitro and in vivo.[35,36] Interestingly, here, we show that A-chain analogue **3** confirms that parts of insulin can keep substantial biological activity. Taken together, our results strongly suggest that the A-chain analogue **3** could offer the possibility to design 'mini-insulin' molecules capable of mimicking insulin effects and bearing some practical advantages for future treatment of diabetes.

Conclusion

We described here the total synthesis of a novel insulin A-chain variant. This analogue of insulin A-chain has been tested in cell culture assays and was found to be as efficient as insulin on DNA synthesis, glucose uptake and glycogen formation. The implication of these data on structure–function relationships of insulin is discussed in the light of already existing body of information in that domain. Investigation on the 3-D structure of analogue **3** is underway and will provide additional knowledge on the mechanism of action of this 'mini-insulin'. However, the present study strongly suggests for the first time that a fragment of insulin can exert similar biological activity as the whole molecule. Such a peptide structure may help understanding the cell binding mechanism of the hormone and provide new perpectives for clinical treatment of diabetes.

Experimental

Materials

Protected Fmoc-amino acids derivatives were purchased from Flandre Chimie (Villeneuve d'Ascq, France) and the SASRIN-TM® resin was from BACHEM (Voisins-le-Bretonneux, France). Chemical reagents used in the FastMoc solid phase synthesis such as 0.5 M 1-hydroxybenzotriazole (HOBt) in *N*,*N*-dimethylformamide (DMF), 2 - (1H - benzotriazol - 1 - yl) - 1,1,2,3 - tetramethyluronium hexafluorophosphate (HBTU) and 2 M diisopropylethylamine (DIEA) in *N*-methylpyrrolidone (NMP) were obtained from Applied Biosystems (Courtaboeuf, France). All other organic solvents and chemical reagents including piperidine, trifluoroacetic acid (TFA), triethylamine, triisopropylsilane (TIS), thioanisole, 1,2-ethanedithiol (EDT), phosphate buffers were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, Saint Quentin Fallavier, France) and used without further purification. Deuterated dimethyl sulfoxide (DMSO-d_6) was from CEA (Saclay, France). Analogue **3** was purified by semi-preparative RP-HPLC and characterized by analytical RP-HPLC on a Thermo-Separation Product Type P200 system controller under gradient elution (detection at 220 nm, manual injection) using an Aquapore ODS Brownlee C18 column (20 μ particle size, 250×10 mm, flow = 3 mL/min) and an Aquapore OD 300 Brownlee C18 column (7 μ particle size, 250×4.6 mm, flow = 1 mL/min), respectively. The molecular weight (M_r) of the human insulin A-chain derivatives (before and after air oxidation) was characterized by electrospray ionization spectroscopy with a Finnigan SSQ710 mass spectrometer (Laboratoire de Technologie Enzymatique, Université de Technologie de Compiègne, France).

Glycogen, dexamethasone, 3-isobutyl-1-methylxanthine, bovine serum albumine (BSA), trichloroacetic acid (TCA) were from Sigma-Aldrich (L'Isle d'Abeau Chesnes, St Quentin Fallavier, France). Media and serum were from Gibco BRL and [^{3}H]-2-deoxy-D-glucose (6 Ci/mmol), [^{3}H]-thymidine (87.5 Ci/mmol), [^{3}H]-D-glucose (21 Ci/mmol) were purchased from NEN (Paris, France). The radioactivity was determined using a liquid scintillation counter 1600TR Packard (Packard, Rungis, France).

^{1}H Nuclear magnetic resonance (NMR) spectroscopy

The peptide was dissolved in DMSO-d_6 at a peptide concentration of 2 mM. The spin systems were assigned at 290 °K, using the DMSO as internal reference and applying the standard method developed by Wütrich.[37] All NMR experiments were carried out on a Bruker AVANCE 500 MHz. Two-dimensional TOCSY (mixing time = 110 ms) and NOESY (mixing time = 400 ms) spectra were acquired with 1024 data points, 256 t1 increment values and 64 scans while 2QF-COSY spectra were recorded with 2048 data points, 512 t1 increments, and 64 scans. All 2-D NMR experiments used frequency discrimination by time-proportional incrementation (TPPI).[38] Prior to Fourier transformation, the time-domain data were multiplied with a shifted sine bell window function and extended by zero filling. Data were processed using the GIFA software developed by the Delsuc group on an SGI r4400 Indigo2 workstation.[39]

FastMoc solid-phase synthesis of bis(thiol) peptide 2

Peptide **2** was synthesized on a SASRIN resin® (333 mg, 0.3 mmol/g, synthesis scale = 0.1 mmol) by solid-phase synthesis using an Applied Biosystem 433A peptide synthesizer according to the manufacturer's instructions.[40,41] The following steps were performed automatically: Fmoc protective group removal was achieved using piperidine in NMP; activating of amino acids was realized using the HBTU/HOBt/DMF/DIEA mixture in NMP; each coupling reaction was obtained with 10 equiv of amino acids and the resin was washed with NMP after each Fmoc removal or coupling step. The reactive side chains of the amino acids were protected using the Trt group (Cys, Asn and Gln residues) and the *t*Bu group (Ser, Thr, Glu and Tyr residues). Then, the protected peptide-resin (150 mg, 0.023 mmol) was treated with 6 mL of TFA/CH_2Cl_2/TIS/H_20/EDT/thioanisole (10:5:1:1:1:1) at room temperature (rt) for 4 h. The mixture was filtered, evaporated and precipitated in cold diethyl ether under argon to provide the crude bis(thiol) peptide **2**. A small portion of the solid peptide was characterized by electrospray ionization spectroscopy (measured mean M_r = 2351.4, calculated value = 2351.6 for $C_{99}H_{155}N_{25}O_{37}S_2$).

Preparation of human insulin A-chain analogue 3 using air oxidation

The crude peptide (**2**) was dissolved in 10 mM freshly prepared phosphate buffer (12 mL, pH = 7.8) at a peptide concentration of 2 mM and triethylamine (10 equiv, 32 μL) was added.[20,22,23] The disulfide bond formation was followed by analytical RP-HPLC. After stirring for 48 h at rt in the presence of atmospheric oxygen, the peptide was purified by semi-preparative RP-HPLC without further treatment and lyophilized. This procedure provided 14 mg of the insulin A-chain analogue **3** (overall yield calculated starting from the resin = 26%) after RP-HPLC purification (100% H_20, 0.1% TFA→60% acetonitrile 0.1% TFA, in 40 min). Characterizations: (i) electrospray ionization spectroscopy: measured mean M_r = 2349.2, calculated value = 2349.4 for $C_{99}H_{153}N_{25}O_{37}S_2$; (ii) analytical RP-HPLC (100% H_2O, 0.1% TFA→60% acetonitrile 0.1% TFA, in 40 min): t_R = 18.08 min; (iii) ^{1}H NMR (500 MHz, DMSO-d_6, ppm δ relative to DMSO): Gly^1 7.92 (1H, H_{NH}, b), 3.58 (2H, H_α, m). Ile^2 8.41 (1H, H_{NH}, d), 4.37 (1H, H_α, m), 1.87 (1H, H_β, m), 1.70 (1H, H_γ, m), 1.41 (1H, H_γ, m), 1.04 (3H, H_γ, m), 0.82 (3H, H_δ, m). Val^3 8.05 (1H, H_{NH}, d), 4.17 (1H, H_α, m), 1.96 (1H, H_β, m), 0.85 (6H, H_γ, m). Glu^4 8.16 (1H, H_{NH}, d), 4.11 (1H, H_α, m), 1.91 (1H, H_β, m), 1.80 (1H, H_β, m), 2.19 (2H, H_γ, m). Gln^5 7.98 (1H, H_{NH}, d), 7.28 (1H, $H_{NH\varepsilon}$, s), 6.81 (1H, $H_{NH\varepsilon}$, s), 4.28 (1H, H_α, m), 1.88 (1H, H_β, m), 1.68 (1H, H_β, m), 2.07 (2H, H_γ, m). Cys^6 8.31 (1H, H_{NH}, d), 4.72 (1H, H_α, m), 3.26 (1H, H_β, m), 2.97 (1H, H_β, m). Ser^7 8.59 (1H, H_{NH}, d), 4.36 (1H, H_α, m), 3.84 (1H, H_β, m), 3.65 (1H, H_β, m). Thr^8 7.51 (1H, H_{NH}, d), 4.28 (1H, H_α, m), 4.22 (1H, H_β, m), 1.10 (3H, H_γ, m). Ser^9 8.16 (1H, H_{NH}, d), 4.17 (1H, H_α, m), 3.60 (2H, H_β, m). Ile^{10} 7.33 (1H, H_{NH},

d), 4.17 (1H, H_α, m), 1.80 (1H, H_β, m), 1.41 (2H, H_γ, m), 1.05 (3H, H_γ, m), 0.82 (3H, H_δ, m). Cys^{11} 8.16 (1H, H_{NH}, d), 4.53 (1H, H_α, m), 3.14 (1H, H_β, m), 2.98 (1H, H_β, m). Ser^{12} 7.84 (1H, H_{NH}, d), 4.34 (1H, H_α, m), 3.57 (1H, H_β, m), 3.53 (1H, H_β, m). Leu^{13} 8.14 (1H, H_{NH}, d), 4.12 (1H, H_α, m), 1.52 (2H, H_β, m), 1.36 (1H, H_γ, m), 1.81 (6H, H_δ, m). Tyr^{14} 7.80 (1H, H_{NH}, d), 6.99 (2H, H_δ, d), 6.60 (2H, H_ε, d), 4.39 (1H, H_α, m), 2.90 (1H, H_β, m), 2.63 (1H, H_β, m). Gln^{15} 8.09 (1H, H_{NH}, d), 7.34 (1H, $H_{NH\varepsilon}$, s), 6.83 (1H, $H_{NH\varepsilon}$, s), 4.24 (1H, H_α, m), 1.88 (1H, H_β, m), 1.77 (1H, H_β, m), 2.11 (2H, H_γ, m). Leu^{16} 7.94 (1H, H_{NH}, d), 4.18 (1H, H_α, m), 1.64 (2H, H_β, m), 1.46 (1H, H_γ, m), 0.85 (6H, H_δ, m). Glu^{17} 8.16 (1H, H_{NH}, d), 4.11 (1H, H_α, m), 1.91 (1H, H_β, m), 1.80 (1H, H_β, m), 2.19 (2H, H_γ, m). Asn^{18} 8.04 (1H, H_{NH}, d), 7.38 (1H, $H_{NH\delta}$, s), 6.95 (1H, $H_{NH\delta}$, s), 4.51 (1H, H_α, m), 2.51 (1H, H_β, m), 2.31 (1H, H_β, m). Tyr^{19} 7.88 (1H, H_{NH}, d), 6.99 (2H, H_δ, d), 6.60 (2H, H_ε, d), 4.40 (1H, H_α, m), 2.95 (1H, H_β, m), 2.67 (1H, H_β, m). Ser^{20} 8.11 (1H, H_{NH}, d), 4.28 (1H, H_α, m), 3.58 (2H, H_β, m). Asn^{21} 8.04 (1H, H_{NH}, d), 7.42 (1H, $H_{NH\delta}$, s), 6.95 (1H, $H_{NH\delta}$, s), 4.51 (1H, H_α, m), 2.51 (1H, H_β, m), 2.31 (1H, H_β, m).

DNA synthesis assay

Assays were performed as previously described.[24] Undifferentiated 3T3-L1 fibroblasts were seeded in 24-well plates (20,000 cells/well) and maintained in DMEM + 10% FCS until 60% confluency. Then, the cells were incubated for 20 h with 500 μL DMEM containing insulin or analogue **3** and labeled with 0.5 μCi/mL [^{3}H]-thymidine for the last 4 h of incubation. At the end of the incubation, the media were removed and the cell layers were fixed and rinsed three times with cold 5% TCA. The precipitated material was then solubilized with 0.1 M NaOH and counted in a liquid scintillation counter.

Differentiation process of 3T3-L1 cells

Assays were performed as previously described.[42,43] 3T3-L1 cells were seeded in six-well plates and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) until confluency. Two days later, cells were fed with DMEM + 10% FCS containing 4.5 g/L glucose, 170 nM insulin, 0.25 μM dexamethasone and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) for 48 h. Then, the medium was removed and replaced every two days with DMEM + 10% FCS containing 4.5 g/L glucose and 170 nM insulin. By day 7, more than 90% of the cells displayed the adipocyte phenotype as reflected by accumulated lipid droplets. The cultures were used for experiments between days 7 and 10.

Glucose uptake

Assays were performed as previously described.[15] Briefly, differentiated 3T3-L1 adipocytes in six-well plates were incubated twice for 30 min in DMEM + 10% FCS, containing 25 mM glucose and 2 mM glutamine without insulin, and once in Kreb's Ringer HEPES (KRH): 130 mM NaCl, 2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.5 mM KCl, 10 mM KH_2PO_4, 25 mM *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES) containing 1% BSA and 5 mM glucose. Cells were then incubated with 1 mL of KRH containing 1% BSA and insulin or analogue **3** for 20 min at 37 °C. [^{3}H]-2-deoxy-D-glucose (2 μCi/mL) was added to each dish for 20 min. Uptake was stopped by three washes with ice-cold with KRH and 5 mM glucose. The cells were then solubilized in 1 mL of 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaOH and the radioactivity was determined by scintillation counting.

Glycogen synthesis

Assays were performed as previously described.[16,44] HepG2 cells grown in six-well plates were serum-starved for 3 h in Roswell Park Memorial Institute (RPMI 1640): 5 mM glucose, 2 mM glutamine, stimulated with insulin or analogue **3** for 30 min, and incubated with [^{3}H]-D-glucose (2 μCi/mL) for 4 h. Incubations were stopped by three rapid washes on ice with phosphate-buffered saline, cells were solubilized in 1 mL of 0.1 M NaOH, and the samples were boiled for 30 min. Carrier glycogen (2 mg) was added and precipitated with 70% ethanol for 2 h at −80 °C. The samples were centrifuged at 3500*g*, the pellet was washed with ethanol, and resuspended in 500 μL of water. Radioactivity of the samples was determined by scintillation counting. Total amount of protein was determined by the method of Bradford.[45] Radioactivity values were corrected to account for differences in the total protein concentration.

Statistical evaluations

Mean values of triplicate or quadruplicate experimental determinations are given with the standard deviation (SD). The statistical analysis of group were performed using unpaired Student's *t*-tests ($*p<0.05$, $**p<0.01$) after having verified that variancies were homogenous in the group. The Student's *t*-test was determined using the control group as a reference where cells were treated without insulin or analogue **3** in the same experimental conditions.

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