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(54) Title: NOVEL INSULIN DERIVATIVES

(57) Abstract

Insulin derivatives with organ preferential action having Tyr in position A13 and/or having Phe, Trp or Tyr in position B17 are provided. Also provided are pharmaceutical preparations containing such insulin derivatives.

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Novel insulin derivatives

Field of this invention

The present invention relates to novel insulin derivatives having tyrosine in position A13 and/or having phenylalanine, tryptophane or tyrosine in position B17, pharmaceutical preparations containing such insulin derivatives, and a process for preparing the insulin derivatives.

Background of the art

Insulin is a hormone which regulates the blood glucose level by decreasing glucose outflow from the liver and by increasing glucose uptake in peripheral tissues, for example muscles and adipose tissues. Insulin exerts these effects by interacting with insulin receptors present on most cells. The insulin receptors on the hepatocytes bind most of the insulin to regulate the metabolism and synthesis of glucose in the liver cells. Insulin reaches the insulin receptors in the peripheral tissues after transendothelial transport, which is fully or partly receptor mediated. This means that insulin has to bind to a receptor on the endothelial cell before it can be transported across this barrier to reach the distal tissues. After having reached the target organs in the periphery, insulin acts among other things by facilitating glucose uptake. Thus, the total reduction of blood glucose by insulin is due to such effects both in the liver and peripheral tissues.

In normal man, pancreatic insulin is secreted directly into the hepatic portal vein, thereby insulinizing the liver and avoiding persistent peripheral hyperinsulinaemia.

In Type I diabetics who lack endogenous insulin secretion, exogenous insulin must be delivered parenterally, and although the most physiological route is delivery of insulin directly into the hepatic portal vein, this is anatomically difficult. Therefore, the subcutaneous route has been used in clinical practice. However, peripheral hyper-

insulinaemia results from the fact that insulin is delivered subcutaneously rather than intraportally, so that the insulin delivered reaches the peripheral tissue first rather than after passage through the liver. This route of administration may, therefore, result in liver hypoinsulinaemia leading to a non-physiological regulation of the diabetic patients.

Type II diabetes is characterized by an excessive endogenous insulin secretion. Due to the high concentration of secreted insulin, the number of insulin receptors on the cell surfaces are decreased. The decrease of insulin receptors will, however, result in a decrease in the diabetics' sensitivity to insulin because this (among other things) is a function of the number of insulin receptors on the individual cells.

One important therapy of Type II diabetes is a diet and weight reduction. Treatment with oral hypoglucaemic drugs is another well established therapy. In certain instances it might, however, be desirable to treat Type II diabetics with insulin.

In order to diminish possible long-term complications arising from peripheral hyperinsulineamia, it would be highly desirable to develop an insulin which primarily has an influence on the glucose production in the liver. It is also desired that the insulin concentration in liver and periphery will more closely resemble the physiological route of insulin delivery.

Insulin derivatives with remarkably high association rate constants will be distributed to the liver to a higher degree than to the peripheral tissues and such derivatives will be of potential value both in the treatment of Type I and Type II diabetes.

Therefore, there is a need in the art for insulin derivatives which after subcutaneous injection are targeted to special organs, for instance the liver and/or muscles.

Insulin derivatives wherein one or more of the amino acid residues present in positions A8, A9, A10, A13,

A21, B1, B2, B5, B9, B10, B12, B14, B16, B17, B18, B20, B26, B27 and B28 are the amino acid residues of human insulin or the same or different amino acid residue substitutions, the net function of which is to impart to the molecule the same charge or a greater negative charge at neutral pH than that of human insulin, are in principle covered by formula I in a European patent application having publication No. 214,826. More specifically, it is stated in this patent applicaion that the A13 amino acid residue can be Pro, Val, Arg, His, Ala, Glu, Asp, Thr, Gly, Gln, Asn or Asp and that the B17 amino acid residue can be Ser, Thr, Asn, Gln, Glu, Asp or His. It is stated that these known insulin derivatives have a rapid onset of effect.

Insulin analogues stated to be hepatospecific are described in International Patent Application having publication No. WO 90/12814. In these known analogues, the amino acid residues in the positions A13, A14, A15, A19 and B16 have been exchanged. There is no mentioning of the B17 position therein. The only specific insulin compounds stated therein are Leu^{A10},Trp^{A14} human insulin and Trp^{A14} sheep insulin (being Ala^{A8},Gly^{A9},Val^{A10},Trp^{A14},Ala^{B30} human insulin).

International patent application having publication No. WO 92/00322 was published after the effective filing date of the present application.

Summary of the invention

This invention is based on the surprising fact that certain insulin derivatives have remarkably fast association rate constants in the insulin receptor binding process.

The insulin derivatives of this invention have tyrosine in position A13 and/or have phenylalanin, tryptophane or tyrosine in position B17. Apart from these alterations, compared with human insulin, the insulin derivatives of this invention may in further positions differ from human insulin. Compared with human insulin, such alterations comprise:

a) compared with human insulin, exchanging the A21 amino acid with another amino acid which can be coded for by nucleotide sequences, for example by Ala, Gln, Glu, His, Ile, Leu, Met, Gly, Ser, Thr, Trp, Tyr or Val, or with hSer, and/or

b) compared with human insulin, exchanging one or more of the amino acids present in positions A4, A17, B13 and B21 with a neutral amino acid which can be coded for by nucleotide sequences, and/or

c) compared with human insulin, exchanging the B27 amino acid with Arg, Lys or Thr and/or

d) omitting 3, 4 or 5 of the amino acid residues in positions B26, B27, B28, B29 and B30 and/or

e) protecting the carboxy group in the amino acid residue in the C terminal end of the B chain with an amino group and/or

f) exchanging the B25 amino acid with tyrosine and/or

g) exchanging the A13 amino acid with phenylalanin or tryptophane and/or

h) omitting the B1 amino acid and/or

i) compared with human insulin, having one or more of the amino acid residues in positions A8, A9, A10, A13, A21, B1, B2, B5, B9, B10, B12, B14, B16, B17, B18, B20, B26, B27 and B28 exchanged with another amino acid residue, the net function of which is to impart to the molecule the same charge or a greater negative charge at neutral pH than that of human insulin.

Preferably, only one or two of these exchanges are made.

The insulin derivatives of this invention have interesting pharmacological properties. For example, the insulin derivatives of this invention are capable of being targeted to special organs after subcutaneous administration.

The insulin derivatives of this invention will thus be of potential value both in the treatment of Type I and Type II diabetes.

The insulin derivatives of this invention are a selected, novel group of insulin derivatives having additio-

nal advantageous characteristics over the known insulin derivatives.

Examples of amino acids which can be coded for by necleotide sequences are Lys, Arg, Gly, Val, Ile, Leu, Phe, Tyr, Met, Asp, Glu, Ala, Ser and Thr.

Examples of neutral amino acids are Gly, Val, Ile, Leu, Phe, Tyr, Met, Asp, Glu, Ala, Ser and Thr.

This invention is also related to novel pharmaceutical preparations containing the above insulin derivatives in a solution with conventional additives, adjuvants, carriers and diluents used for known insulin preparations.

The insulin derivatives of this invention may be prepared by chemical synthesis by methods analogous to the method described by Märki et al. (Hoppe-Seyler's Z. Physiol.Chem., 360 (1979), 1619 - 1632). They may also be formed from separately in vitro prepared A and B chains containing the appropriate amino acid residue substitutions, whereupon the modified A and B chains are linked together by establishing disulphide bridges according to known methods (for example Chance et al., In: Rick, D.H., and Gross, E., (editors) Peptides: Synthesis - Structure - Function. Proceedings of the seventh American peptide symposium, Illinois, pp. 721 - 728).

A more preferred method could be to make the insulin derivative biosynthetically. Thus, the insulin derivatives of this invention may, for example, be prepared by altering the proinsulin gene through replacement of codon coding for Leu in position A13 and/or in position B17, in the native human proinsulin gene by codon(s) encoding the desired amino acid residue substitute(s) or by synthesizing the whole DNA-sequence encoding the desired insulin derivative. The gene encoding the desired insulin derivative is then inserted into a suitable expression vector which when transferred to a suitable host organism, for example E. coli, Bacillus or yeast, generates the desired product. The product expressed is then isolated from the cells or the

culture broth depending on whether the expressed product is secreted from the cells or not.

The insulin derivatives of this invention may, furthermore, be prepared by a method derivative to the method described in European patent application having publication No. 195,691, the disclosure of which is incorporated by reference hereinto. By such a method, an insulin derivative precursor of human insulin wherein Lys^{B29} is connected to Gly^{A21} by means of either a peptide bond or a peptide chain of varying length, is expressed and secreted by yeast and then converted into human insulin by the so-called transpeptidation reaction.

Accordingly, the insulin derivatives of this invention may be prepared by inserting a DNA-sequence encoding a precursor of the insulin derivative in question into a suitable yeast expression vehicle which when transferred to yeast is capable of expressing and secreting the precursor of the insulin derivative in which Lys^{B29} is connected to Gly^{A21} by a peptide bond or a peptide chain with the general formula I

$$-R_n-R'- \qquad (I)$$

wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33, and R' is Lys or Arg, when the transformed yeast strain is cultured in a suitable nutrient medium. The precursor is then recovered from the culture broth and reacted with an amino compound with the general formula II

$$Q\text{-}OR'' \qquad (II)$$

wherein Q is the amino acid residue which is to be inserted in the B30 position, preferably Thr, and R" is a carboxy protecting group (for example methyl or tert.butyl), using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents analogously as described in US

patent specification No. 4,343,898, the disclosure of which is incorporated by reference hereinto. Thereafter, the carboxy protecting group is removed and the insulin derivative is isolated from the reaction mixture.

The insulin derivatives of this invention may also be prepared by a method analogous to the method described in European patent application having publication No. 195,691 the disclosure of which is incorporated by reference hereinto. By this method, insulin derivative precursors of the type having a bridge between the A and B chain consisting of a single pair of basic amino acid (Lys or Arg) are produced in yeast and then converted into the insulin derivative by an enzymatic conversion.

The insulin derivatives of this invention may be used for the preparation of novel insulin preparations. Such novel insulin preparations may contain the insulin derivatives of this invention or a pharmaceutically acceptable salt thereof in aqueous solution or suspension, preferably at an approximately neutral pH value. The aqueous medium is made isotonic, for example with sodium chloride, sodium acetate or glycerol. Furthermore, the aqueous medium may contain zinc ions, buffers such as acetate and citrate and preservatives such as m-cresol, methylparaben or phenol. The pH value of the preparation is adjusted to the desired value. The insulin preparation is made sterile by sterile filtration.

Examples of insulin derivatives of this present invention are Tyr^{A13} human insulin, Phe^{B17} human insulin, Trp^{B17} human insulin, Tyr^{B17} human insulin, Tyr^{A13},Phe^{B17} human insulin, Tyr^{A13},Trp^{B17} human insulin, Tyr^{A13},Tyr^{B17} human insulin, Phe^{A13},Phe^{B17} human insulin, Phe^{A13},Trp^{B17} human insulin, Phe^{A13},Tyr^{B17} human insulin, Trp^{A13},Phe^{B17} human insulin, Trp^{A13},Trp^{B17} human insulin and Trp^{A13},Tyr^{B17} human insulin.

Terminology

The abbreviations used for the amino acids are those stated in J.Biol.Chem. 243 (1968), 3558. The amino acids are in the L configuration. Unless otherwise indicated, the species of insulins stated herein is human.

The replacement(s) made in the human insulin molecule according to the practice of this invention is (are) indicated with a prefix referenced to human insulin. As an example, Glu^{A13} human insulin is human insulin having Glu in position 13 of the A chain (in stead of Leu).

Like other insulins, the insulin derivatives of this invention have a low toxicity.

The insulin derivatives of this invention are administered analogously to the administration of known insulins and insulin derivatives in a therapeutically effective amount. The dosage to be administered is normally determined by a physician. The route of administration may be intramuscular, subcutaneously, intravenous, via a mucos, for example nasal, or by a pump.

Any novel feature or combination of features described herein is considered essential to this inverntion.

Detailed description

Genes encoding the precursors of the insulin derivative can be prepared by modification of genes encoding the corresponding human insulin precursors by site specific mutagenesis to insert or substitute with codons encoding the desired mutation. A DNA-sequence encoding the precursor of the insulin derivative may also be made by enzymatic synthesis from oligonucleotides corresponding in whole or part to the insulin derivative precursor gene.

DNA-sequences containing a gene with the desired mutation are then combined with a suitable promoter sequence, for example fragments coding for the TPI promoter (TPIp) (T. Alber and G. Kawasaki, Nucleotide Sequence of the triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol.Applied Genet. 1 (1982), 419 - 434), a suitable leader

sequence and possible transcription termination sequence, for example from TPI of S. cerevisiae (TPI_T). These fragments provide sequences to ensure a high rate of transcription of the precursor encoding gene and also provide a presequence which can effect the localization of the precursor into the secretory pathway and its eventual excretion into the growth medium. The expression units are furthermore provided with a yeast origin of replication, for instance the 2μ origin, and a selectable marker, for instance LEU 2.

The selected plasmid is then transformed into a suitable yeast strain by conventional technique, for example as described in European patent application having publication No. 214,826 and transformants are grown on YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The insulin derivative precursor is isolated from the culture medium and reacted with threonine methyl ester acetate dissolved in a N,N-dimethylformamide/water mixture in the presence of trypsin as described in European patent application having publication No. 214,826 and converted into the human insulin derivative by acidic or basic hydrolysis, see European patent application having publication No 214,826.

Example 1

The specificity of a compound according to this invention, i.e. Phe^{B17} human insulin, compared with human insulin was determined by the following method which appeared in Diabetes May 1991:

Insulin and insulin derivative were monoiodinated in the tyrosine in the A14 position with ^{123}I. Wistar rats weighing about 250 g received a bolus injection of 5 - 10 mU of ^{123}I-labelled insulin derivative through a catheter inserted into the jugular vein. The liver activity of ^{123}I was monitored by a gammacamera and expressed as percentage of total radioactivity. Results are shown in Table I below wherein "HI" designates human insulin.

Table I

LIVER ACTIVITY

Time, minutes	$Phe^{B17}HI$	HI
1	40	33
2	50	40
3	54	42
4	54	44
5	50	41
6	48	40
7	42	35
8	38	32
9	35	30
10	31	26

As appears from this table, the compound of this invention is preferentially targeted to the liver.

CLAIMS

1. Insulin derivatives differing from human insulin in that they have Tyr in position A13 and/or having Phe, Trp or Tyr in position B17.

2. Insulin derivative according to claim 1 having Phe, Trp or Tyr in position B17.

3. Derivative according to claims 1 or 2 having - compared with human insulin - the amino acid residue in position A21 exchanged with a residue of another naturally occurring amino acid.

4. Derivative according to any of the preceding claims having - compared with human insulin - one or more of the amino acid residues in positions A4, A17, B13 and B21 exchanged with a neutral amino acid which can be coded for by nucleotide sequences.

5. Derivative according to any of the preceding claims having - compared with human insulin - the amino acid residue in position B27 exchanged with Arg, Lys or Thr.

6. Derivative according to any of the preceding claims wherein 3, 4 or 5 of the amino acid residues in positions B26, B27, B28, B29 and B30 are omitted.

7. Derivative according to any one of the preceding claims having the carboxy group in the amino acid residue at the C terminal end of the B chain protected with an amino group.

8. Derivative according to any one of the preceding claims wherein the amino acid residue in position B25 is Tyr.

9. Derivative according to any one of the preceding claims wherein the amino acid residue in position A13 is Phe or Trp.

10. Derivative according to any one of the preceding claims having - compared with human insulin - no B1 amino acid residue.

11. Derivative according to any one of the preceding claims having - compared with human insulin - one or

more of the amino acid residues in positions A8, A9, A10, A13, A21, B1, B2, B5, B9, B10, B12, B14, B16, B17, B18, B20, B26, B27 and B28 exchanged with another amino acid residue, the net function of which is to impart to the molecule the same charge or a greater negative charge at neutral pH than that of human insulin.

12. Derivative according to claim 1 being Tyr^{A13} human insulin, Phe^{B17} human insulin, Trp^{B17} human insulin, Tyr^{B17} human insulin, Tyr^{A13},Phe^{B17} human insulin, Tyr^{A13},Trp^{B17} human insulin, Tyr^{A13},Tyr^{B17} human insulin, Phe^{A13},Phe^{B17} human insulin, Phe^{A13},Trp^{B17} human insulin, Phe^{A13},Tyr^{B17} human insulin, Trp^{A13},Phe^{B17} human insulin, Trp^{A13},Trp^{B17} human insulin or Trp^{A13},Tyr^{B17} human insulin.

13. Derivative according to any one of the preceding claims having organ preferential action.

14. Derivative according to Claim 13, wherein the organ is the liver.

15. Pharmaceutical preparations containing an insulin derivative according to any one of the preseding claims or a pharmaceutically acceptable salt thereof and conventional pharmaceutical additives, adjuvants, carriers, diluents and solvents.

16. Preparation according to claim 15 for use in the treatment of diabetes.

17. A method of treating diabetes in a patient in need of such treatment comprising administering to the patient a therapeutically active amount of an insulin derivative as defined in any one of the claims 1 through 14, optionally together with a pharmaceutically acceptable carrier.

18. Any novel feature or combination of features described herein.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00059

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/40, A 61 K 37/26//C 07 K 99:26

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO, A1, 9012814 (MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK) 1 November 1990, see claim 2 and the abstract --	9
A	EP, A2, 0214826 (NOVO INDUSTRI A/S) 18 March 1987, see the whole document --	1-16, 18
P,A	WO, A1, 9200322 (NOVO NORDISK A/S) 9 January 1992, see the whole document --	1-16, 18
A	WO, A1, 8605497 (NORDISK GENTOFTE A/S) 25 September 1986, see the whole document --	4

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th May 1992	1992 -06- 04
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Elisabeth Carlborg

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 9001038 (NORDISK GENTOFTE A/S) 8 February 1990, see the whole document --	13

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 17, because ~~they~~ it is relate to subject matter not required to be searched by this Authority, namely:

Method for treatment of the human or animal body by therapy. Rule 39(iv).

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00059

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 28/03/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9012814	90-11-01	AU-D-	5541590	90-11-16
		CA-A-	2014896	90-10-20
		EP-A-	0469084	92-02-05
EP-A2- 0214826	87-03-18	AU-B-	593274	90-02-08
		AU-D-	6206686	87-03-05
		JP-A-	62053999	87-03-09
WO-A1- 9200322	92-01-09	AU-D-	8008091	92-01-23
WO-A1- 8605497	86-09-25	AU-B-	596968	90-05-24
		AU-D-	5622686	86-10-13
		AU-D-	5622786	86-10-13
		EP-A-B-	0216832	87-04-08
		EP-A-	0216833	87-04-08
		JP-T-	62502196	87-08-27
		JP-T-	62502538	87-10-01
		US-A-	5028586	91-07-02
		WO-A-	86/05496	86-09-25
WO-A1- 9001038	90-02-08	EP-A-	0425482	91-05-08
		ES-A-	2010299	89-11-01