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DESCRIPTION

Technical Field

[0001] The present invention relates to a glucagon derivative, a long-acting conjugate of the glucagon derivative, and a use thereof.

Background Art

[0002] Due to recent economic growth and changes in dietary habits, etc., the incidence of metabolic syndrome-associated diseases including various diseases such as obesity, hyperlipidemia, hypertension, arteriosclerosis, hyperinsulinemia, diabetes, and liver diseases is rapidly increasing. These diseases may occur independently but in general they mostly occur in close relationship with each other, being accompanied by various symptoms.

[0003] In particular, according to the World Health Organization (WHO), more than one billion adults are overweight worldwide, among them over 3 million are clinically obese, and 250,000 people die every year in Europe and more than 2.5 million people worldwide die every year due to overweight-related diseases.

[0004] Overweight and obesity are responsible for increasing blood pressure and cholesterol levels and causing or worsening various diseases, such as cardiac diseases, diabetes, arthritis, etc. In addition, the problem of obesity is also becoming a major cause in the increased incidence of arteriosclerosis, hypertension, hyperlipidemia, or heart diseases in children or teenagers as well as in adults.

[0005] Although obesity is a severe condition that causes various diseases worldwide as described above, it is thought to be overcome by individual effort, and it is also believed that obese patients lack self-control. However, obesity is not easy to treat, because it is a complex disease associated with the mechanisms of appetite control and energy metabolism. Accordingly, the treatment of obesity requires not only the efforts of obese patients, but also a method capable of treating abnormal mechanisms associated with appetite control and energy metabolism. Thus, efforts have been made to develop drugs for treating the abnormal mechanisms.

[0006] As a result of these efforts, drugs such as Rimonabant[®] (Sanofi-Aventis), Sibutramin[®] (Abbott), Contrave[®] (Takeda), Orlistat[®] (Roche), etc have been developed, but they have the disadvantages of serious adverse effects or very weak anti-obesity effects. For example, according to a report, Rimonabant[®] shows a side-effect of central nervous system disorder, Sibutramine[®] and Contrave[®] show cardiovascular side-effects, and Orlistat[®] shows only about

4 kg of weight loss when taken for one year. Accordingly, there are no therapeutic agents for obesity which can be prescribed safely for obese patients.

[0007] Many extensive studies have been made to develop novel therapeutic agents for obesity which can resolve the problems of the conventional anti-obesity drugs. Recently, glucagon derivatives have received much attention. Glucagon is produced by the pancreas when blood glucose levels drop as a result of other medications or diseases, or hormone or enzyme deficiencies. Glucagon sends a signal for glycogen breakdown in the liver and a subsequent glucose release and plays a role in increasing blood glucose levels to a normal range. In addition to the effect of increasing the blood glucose levels, glucagon suppresses appetite and activates hormone-sensitive lipase of adipocytes to facilitate lipolysis, thereby showing an anti-obesity effect. However, the use of glucagon as a therapeutic agent has been limited because it has a low solubility and it is precipitated at a neutral pH.

[0008] Accordingly, the glucagon with improved properties alone can be effectively used for the treatment of severe hypoglycemia, nonalcoholic steatohepatitis (NASH), dyslipidemia, etc., due to its activities of fat decomposition and β -oxydation in the liver.

[0009] One of the glucagon derivatives, glucagon-like peptide-1 (GLP-1), is under development as a therapeutic agent for treating hyperglycemia in patients with diabetes. GLP-1 has the functions of stimulating insulin synthesis and secretion, inhibiting glucagon secretion, slowing gastric emptying, increasing glucose utilization, and inhibiting food intake.

[0010] Exendin-4, prepared from lizard venom and having an amino acid homology of about 50% with GLP-1, was also reported to activate the GLP-1 receptor, thereby improving hyperglycemia in patients with diabetes (J Biol Chem. 1992 Apr 15; 267 (11): 7402-5). However, anti-obesity drugs containing GLP-1 are reported to show side-effects such as vomiting and nausea.

[0011] As an alternative to GLP-1, therefore, much attention has been focused on oxyntomodulin, which can bind to both receptors of the two peptides, GLP-1 and glucagon. Oxyntomodulin is a peptide prepared from a glucagon precursor, pre-glucagon, and has the functions of inhibiting food intake and enhancing satiety of GLP-1, and has lipolytic activity like glucagon, thus increasing its potency in anti-obesity therapy.

[0012] However, oxyntomodulin or derivatives thereof have a serious drawback in that an excess amount of the drug should be administered daily because they have low efficacy and a short *in vivo* half-life.

[0013] Additionally, when both activities of GLP-1 and glucagon are present in a single peptide, the activity ratio thereof becomes fixed, and thus it is difficult to use a dual agonist with various ratios. Accordingly, a combined therapy capable of using various activity ratios by adjusting the contents of GLP-1 and glucagon may be more effective. However, for the combined therapy, it is required to improve the physical characteristics of glucagon, which aggregates at a neutral

pH and precipitates with time, thus showing poor solubility.

[0014] EP 3241841 A1 belongs to the state of the art under Article 54(3) EPC and discloses glucagon derivative peptides and their use in the prevention and treatment of hypoglycemia and obesity.

[0015] WO 2012/169798 A2 discloses GLP-1 receptor agonists for use in the treatment of obesity, used in combination with other compounds having a therapeutic activity against metabolic syndrome.

[0016] WO 2011/075393 A2 discloses glucagon derivatives which are glucagon/GLP-1 receptor co-agonists and related conjugates, pharmaceutical compositions, and uses thereof.

[0017] WO 2014/073842 A1 discloses a long-lasting oxyntomodulin-immunoglobulin Fc conjugate.

[0018] Under these circumstances, the present inventors have developed glucagon derivatives with partial modifications of the amino acid sequence of glucagon for improving the therapeutic effects of glucagon on hypoglycemia and obesity by improving the physical properties of glucagon, and have discovered that these glucagon derivatives, due to the altered pI values which are different from that of native glucagon, have improved solubility and higher stability at a neutral pH and have confirmed that the developed glucagon derivative activates its receptors in *in vitro* assay, thereby completing the present invention.

Disclosure of Invention

Technical Problem

[0019] An object of the present invention is to provide a pharmaceutical composition for treating or preventing metabolic syndrome, containing

i) a peptide comprising the amino acid sequence of SEQ ID NO: 20 or 37 and ii) at least one compound or material having a therapeutic activity for metabolic syndromean insulinotropic peptide, wherein the peptide comprising the amino acid sequence of SEQ ID NO: 20 or 37 is in the form of a long-acting conjugate, to which a biocompatible material capable of increasing in vivo half-life of the peptide is linked, and the insulinotropic peptide is in the form of a long-acting conjugate, to which a biocompatible material capable of increasing in vivo half-life of the insulinotropic peptide is linked.

[0020] Another object of the present invention is to provide an novel glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37.

[0021] Still another object of the present invention is to provide an isolated polynucleotide encoding the glucagon derivative, a vector including the polynucleotide, and an isolated cell including the polynucleotide or the vector.

[0022] Still another object of the present invention is to provide an isolated conjugate in which the glucagon derivative and a biocompatible material which is capable of increasing *in vivo* half-life are linked.

[0023] Still another object of the present invention is to provide a composition containing the glucagon derivative and the isolated conjugate.

[0024] Still another object of the present invention is to provide a pharmaceutical composition for treating or preventing hypoglycemia or metabolic syndrome, containing the glucagon derivative or the isolated conjugate.

Solution to Problem

[0025] The pharmaceutical composition of the invention is defined in the claims.

[0026] In specific embodiment, the peptide comprising the amino acid sequence of SEQ ID NO: 20 or 37 is characterized in that it has a pI value different to that of native glucagon, e.g., a pI of 6.5 or less, or a pI of 7.0 or higher.

[0027] In specific embodiment, the insulinotropic peptide is characterized in that it is selected from the group consisting of GLP-1, exendin-3, exendin-4, an agonist thereof, a derivative thereof, a fragment thereof, a variant thereof, and a combination thereof.

[0028] In still another specific embodiment, the insulinotropic peptide is characterized in that it is an insulinotropic peptide derivative in which the N-terminal histidine residue of the insulinotropic peptide is substituted with one selected from the group consisting of desamino-histidyl, N-dimethyl-histidyl, β -hydroxy imidazopropionyl, 4-imidazoacetyl, and β -carboxy imidazopropionyl.

[0029] In still another specific embodiment, the insulinotropic peptide is characterized in that it is selected from the group consisting of a native exendin-4; an exendin-4 derivative in which the N-terminal amine group of exendin-4 is deleted; an exendin-4 derivative in which the N-terminal amine group of exendin-4 is substituted with a hydroxyl group; an exendin-4 derivative in which the N-terminal amine group of exendin-4 is modified with a dimethyl group; an exendin-4 derivative in which the α -carbon of the 1st amino acid of exendin-4, histidine, is deleted; an exendin-4 derivative in which the 12th amino acid of exendin-4, lysine, is substituted with serine, and an exendin-4 derivative in which the 12th amino acid of exendin-4, lysine, is substituted with arginine.

[0030] In still another specific embodiment, the biocompatible material is characterized in that it is selected from the group consisting of polyethylene glycol, fatty acid, cholesterol, albumin and a fragment thereof, an albumin-binding material, a polymer of repeating units of a particular amino acid sequence, an antibody, an antibody fragment, an FcRn-binding material, in vivo connective tissue or a derivative thereof, a nucleotide, fibronectin, transferrin, a saccharide, and a polymer.

[0031] In still another specific embodiment, the peptide comprising the amino acid sequence of SEQ ID NO: 20 or 37 and the insulinotropic peptide are characterized in that they are respectively linked to a biocompatible material by a linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, fatty acid, a polymer, a low molecular weight compound, a nucleotide, and a combination thereof.

[0032] In still another specific embodiment, the biocompatible material is characterized in that it is an FcRn-binding material, and the peptide comprising the amino acid sequence or SEQ ID NO: 20 or 31 and the insulinotropic peptide are respectively linked to a biocompatible material by a peptide linker or a non-peptide linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, polyvinyl ethyl ether, dextran, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, and a combination thereof.

[0033] In still another specific embodiment, the FcRn-binding material is characterized in that it is a polypeptide including an immunoglobulin Fc region.

[0034] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is aglycosylated.

[0035] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is selected from the group consisting of:

1. (a) a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain;
2. (b) a CH1 domain and a CH2 domain;
3. (c) a CH1 domain and a CH3 domain;
4. (d) a CH2 domain and a CH3 domain;
5. (e) a combination between one or two or more domains among a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain and an immunoglobulin hinge region or a part of the hinge region; and
6. (f) a dimer between each domain of the heavy chain constant region and the light chain constant region.

[0036] In still another specific embodiment, the polypeptide including the immunoglobulin Fc region is in the form of a dimer.

[0037] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is a native Fc derivative in which the region capable of forming a disulfide bond is deleted, a native Fc derivative in which a part of the amino acid(s) in the N-terminus is deleted, a native Fc derivative in which a methionine is added to the N-terminus, a native Fc derivative in which a complement-binding site is deleted, or a native Fc derivative in which an antibody dependent cell mediated cytotoxicity (ADCC) site is deleted.

[0038] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is an Fc region derived from an immunoglobulin selected from the group consisting of IgG, IgA, IgD, IgE, and IgM.

[0039] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is an IgG4 Fc region.

[0040] In still another specific embodiment, the immunoglobulin Fc region is characterized in that it is an aglycosylated Fc region derived from human IgG4.

[0041] In still another specific embodiment, the non-peptide linker is characterized in that it is linked to the cysteine residue of a peptide including the amino acid sequence of SEQ ID NO: 20 or 37.

[0042] In still another specific embodiment, the non-peptide linker is characterized in that both ends of the non-peptide linker are respectively linked to an amine group or a thiol group of a peptide, which comprises the amino sequence of SEQ ID NO: 20 or 37 or an insulinotropic peptide, and a biocompatible material.

[0043] In another aspect, the present invention provides a novel glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37.

[0044] In still another aspect, the present invention provides an isolated polynucleotide encoding the glucagon derivative, a vector including the polynucleotide, and an isolated cell including the polynucleotide or the vector.

[0045] In still another aspect, the present invention provides an isolated conjugate in which a glucagon derivative and a biocompatible material capable of increasing *in vivo* half-life are linked.

[0046] In a specific embodiment, the biocompatible material is characterized in that the biocompatible material is selected from the group consisting of polyethylene glycol, fatty acid,

cholesterol, albumin and a fragment thereof, an albumin-binding material, a polymer of repeating units of a particular amino acid sequence, an antibody, an antibody fragment, an FcRn-binding material, in vivo connective tissue or a derivative thereof, a nucleotide, fibronectin, transferrin, a saccharide, and a polymer.

[0047] In a specific embodiment, the isolated peptide is characterized in that it is linked to a biocompatible material by a linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, fatty acid, a polymer, a low molecular weight compound, a nucleotide, and a combination thereof.

[0048] In a specific embodiment, the biocompatible material is characterized in that it is an FcRn-binding material, and

[0049] the isolated peptide and the insulinotropic peptide are respectively linked to a biocompatible material by a peptide linker or a non-peptide linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, polyvinyl ethyl ether, dextran, a biodegradable polymer such as polylactic acid (PLA) or polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, and a combination thereof.

[0050] In a specific embodiment, the FcRn-binding material is characterized in that it is a polypeptide including an immunoglobulin Fc region.

[0051] In still another aspect, the present invention provides a composition including the glucagon derivative or the isolated conjugate.

[0052] In a specific embodiment, the composition is characterized in that it is a pharmaceutical composition for treating or preventing hypoglycemia or metabolic syndrome.

Advantageous Effects of Invention

[0053] The glucagon derivatives of the present invention has improved physical properties compared to that of native glucagon and thus can be effectively used as a therapeutic agent for treating hypoglycemia by improving the compliance of patients. Additionally, the glucagon derivatives of the present invention can be effectively used for the prevention and treatment of hypoglycemia and metabolic syndrome such as obesity, diabetes, and nonalcoholic steatohepatitis (NASH).

Brief Description of Drawings

[0054]

FIG. 1 shows a graph illustrating the changes in body weight of obesity animal models (rats), which were prepared by high-fat diet, during a single or combined administration of a long-acting insulintropic peptide conjugate (named as a long-acting exendin-4 derivative) and a long-acting glucagon derivative conjugate (named as a long-acting derivative of SEQ ID NO: 12) with an adjusted dose to the rats, at 3-day intervals for 15 days.

FIG. 2 shows a result illustrating the amount of mesenteric fat of obesity animal models (rats), which were prepared by high-fat diet, measured after a single or combined administration of a long-acting insulintropic peptide conjugate (named as a long-acting exendin-4 derivative) and a long-acting glucagon derivative conjugate (named as a long-acting derivative of SEQ ID NO: 12) with an adjusted dose to the rats for 15 days (* $p < 0.05$, ** $p < 0.01$ vs. vehicle by ANOVA test).

FIG. 3 shows a result illustrating the difference in liver weight of obesity animal models (rats), which were prepared by high-fat diet, measured after a single or combined administration of a long-acting insulintropic peptide conjugate (named as a long-acting exendin-4 derivative) and a long-acting glucagon derivative conjugate (named as a long-acting derivative of SEQ ID NO: 12) with an adjusted dose to the rats for 15 days (** $p < 0.01$, *** $p < 0.001$ vs. vehicle by ANOVA test).

FIG. 4 shows a graph illustrating the changes in body weight (BW) of obesity animal models (mice), which were prepared by high-fat diet, after a single or combined administration of a long-acting insulintropic peptide conjugate (named as a long-acting exendin-4 derivative) and a long-acting glucagon derivative conjugate (named as a long-acting derivative of SEQ ID NO: 20) with an adjusted dose to the rats for 22 days.

FIG. 5 shows a result illustrating the changes in cholesterol content in blood of obesity animal models (mice), which were prepared by high-fat diet, after a single or combined administration of a long-acting insulintropic peptide conjugate (named as a long-acting exendin-4 derivative) and a long-acting glucagon derivative conjugate (named as a long-acting derivative of SEQ ID NO: 20) with an adjusted dose to the rats for 22 days.

Best Mode for Carrying out the Invention

[0055] The specific details of the present invention may be explained as follows. In particular, the explanations and embodiments disclosed in the present invention may be applied to other explanations and embodiments, respectively. That is, all combinations of various elements disclosed in the present invention belong to the scope of the present invention. Additionally, the

scope of the present invention should not be limited by the specific descriptions described herein below.

[0056] Throughout the disclosure of the present invention, not only the conventional 1-letter codes and 3-letter codes for amino acids present in nature but also the 3-letter codes, such as Aib (α -aminoisobutyric acid), Sar(N-methylglycine) generally used for other amino acids, are used. Additionally, the amino acids mentioned in abbreviation in the present disclosure are described according to the IUPAC-IUB Nomenclature.

[0057]

alanine A arginine R

asparagine N aspartic acid D

cysteine C glutamic acid E

glutamine Q glycine G

histidine H isoleucine I

leucine L lysine K

methionine M phenylalanine F

proline P serine S

threonine T tryptophan W

tyrosine Y valine V

[0058] A glucagon derivative includes a peptide having at least one difference in the amino acid sequence compared to native glucagon, a peptide in which the sequence of native glucagon is modified by modifying native glucagon, and a native glucagon mimetic that can activate glucagon receptors like native glucagon.

[0059] Such a glucagon derivative may be one having improved physical properties by having an altered pI relative to native glucagon. Additionally, the glucagon derivative may be one with improved solubility while maintaining the activity of activating glucagon receptors, but is not limited thereto.

[0060] Additionally, the glucagon derivative may be a non-naturally occurring glucagon.

[0061] In particular, native glucagon may have the following amino acid sequence:

[0062] His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-

Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr (SEQ ID NO: 1)

[0063] As used herein, the term "pI" or "isoelectric point" refers to the pH value at which a macromolecule such as a polypeptide has no net charge (0). In the case of a polypeptide with various charged functional groups, the net charge of the total polypeptide is "0" at a point where the pH value is the same as that of the pI. The net charge of the polypeptide at a pH higher than the pI will be negative while the net charge of the polypeptide at a pH lower than the pI will be positive.

[0064] The pI values may be determined on an immobilized pH gradient gel consisting of polyacrylamide, starch, or agarose by isoelectric electrophoresis, or may be estimated, for example, from an amino acid sequence using a pI/MW tool (http://expasy.org/tools/pi_tool.html; Gasteiger et al., 2003) in an ExPASy server.

[0065] As used herein, the term "altered pI" refers to a pI which is different from that of native glucagon due to the substitution of a part of the amino acid sequence of native glucagon with an amino acid residue having a negative charge or a positive charge, i.e., a reduced or increased pI value. The peptide with such an altered pI can exhibit improved solubility and high stability at a neutral pH as a glucagon derivative.

[0066] More specifically, a glucagon derivative may have an altered pI value, not the pI value (6.8) of native glucagon, and even more specifically, a pI value of less than 6.8, more specifically, 6.7 or less, more specifically 6.5 or less, and additionally, a pI value exceeding 6.8, 7 or higher, more specifically, 7.5 or higher, when the pI value is different from that of native glucagon a glucagon derivative exhibits an improved solubility at a neutral pH compared to that of native glucagon thus showing a low level of aggregation.

[0067] More specifically, the glucagon derivative may have a pI value of from 4 to 6.5 and/ or from 7 to 9.5, specifically from 7.5 to 9.5, and more specifically, from 8.0 to 9.3, but the pI value is not limited thereto. In this case, due to the lower or higher pI value compared to that of native glucagon, an improved solubility and high stability at a neutral pH compared to that of native glucagon can be exhibited.

[0068] Specifically, a derivative of native glucagon may be modified by any one method of substitution, addition, deletion, and modification, or a combination thereof in part of the amino acid of native glucagon.

[0069] Examples of the glucagon derivatives prepared by a combination of the above methods include a peptide which differs in at least one amino acid residue of the amino acid sequence compared to that of native glucagon and in which the N-terminal amino acid residue is deaminated, having the function of activating a glucagon receptor, but is not limited thereto, and the native glucagon derivatives can be prepared by a combination of various methods for preparing the derivatives.

[0070] Additionally, such modification for the preparation of native glucagon derivatives may include all of the modifications using L-type or D-type amino acids, and/or non-native type amino acids; and/or a modification of native sequence, for example, modification of a functional group, an intramolecular covalent bonding (e.g., a ring formation between side chains), methylation, acylation, ubiquitination, phosphorylation, aminohexanation, biotinylation, etc.

[0071] Additionally, the modification may also include all those where one or more amino acids are added to the amino and/or carboxy terminal of native glucagon.

[0072] During the substitution or addition of amino acids, not only the 20 amino acids commonly found in human proteins, but also atypical or non-naturally occurring amino acids can be used. Commercial sources of atypical amino acids may include Sigma-Aldrich, ChemPep Inc., and Genzyme Pharmaceuticals. The peptides including these amino acids and atypical peptide sequences may be synthesized and purchased from commercial suppliers, e.g., American Peptide Company, Bachem (USA), or Anygen (Korea).

[0073] Since glucagon has a pH of about 7, it is insoluble in a solution having a physiological pH (pH 4 to 8) and tends to precipitate at a neutral pH. In an aqueous solution with a pH of 3 or below, glucagon is dissolved at the initial stage but precipitates within one hour by forming a gel. Since the gelated glucagon mainly consists of β -sheet fibrils, the administration of the thus-precipitated glucagon via an injection needle or intravenous injection will block blood vessels, and thus is not suitable for use as an injection agent. In order to delay the precipitation process, acidic (pH 2 to 4) formulations are commonly used, and by doing so, glucagon can be maintained in a relatively non-aggregated state for a short period of time. However, glucagon can form fibrils very rapidly at a low pH, and thus these acidic formulations must be injected upon preparation.

[0074] In this regard, the present inventors have developed glucagon derivatives with extended action profiles by modifying the pI of native glucagon via substitution of amino acid residues having negative charges and positive charges. The glucagon derivatives of the present invention, by having an altered pI compared to that of native glucagon, is characterized in having improved solubility and/or high stability at a neutral pH, compared to that of native glucagon.

[0075] The glucagon derivative of the present invention is an isolated peptide comprising the amino acid sequence of SEQ ID

[0076] Additionally, although described as "a peptide consisting of a particular SEQ ID NO" in the present invention, such expression does not exclude a mutation in the peptide that can occur by a meaningless sequence addition upstream or downstream of the amino acid sequence of the corresponding SEQ ID NO, or a naturally-occurring mutation therein, or a silent mutation therein, as long as the peptide having such mutation has an activity the same as or corresponding to that of the peptide which consists of an amino acid sequence of the corresponding SEQ ID NO. Even when the sequence addition or a mutation is present, it

obviously belongs to the scope of the present invention.

[0077] the peptide may be modified in its amino terminus or carboxy terminus or protected by various organic groups for protecting the peptide from protein-cleaving enzymes *in vivo* while increasing its stability, for example, one in which its C-terminus is amidated.

[0078] Additionally, the peptide of the present invention may be synthesized by a method well known in the art, according to its length, e.g., by an automatic peptide synthesizer, and may be produced by genetic engineering technology.

[0079] Specifically, the peptide of the present invention may be prepared by a standard synthesis method, a recombinant expression system, or any other method known in the art. Accordingly, the glucagon derivative of the present invention may be synthesized by various methods including, for example, the methods described below:

(a) a method of synthesizing a peptide by a solid-phase or liquid-phase method stepwise or by fragment assembly, followed by isolation and purification of the final peptide product; or

(b) a method of expressing a nucleic acid construct encoding a peptide in a host cell and recovering the expression product from the host cell culture; or

(c) a method of performing an *in vitro* cell-free expression of a nucleic acid construct encoding a peptide and recovering the expression product therefrom; or

a method of obtaining peptide fragments by any combination of the methods (a), (b), and (c), obtaining the peptide by linking the peptide fragments, and then recovering the peptide.

[0080] In a more specific example, a desired glucagon derivative may be produced by genetic manipulation, which includes preparing a fusion gene encoding a fusion protein, including a fusion partner and a glucagon derivative, transforming the resultant into a host cell, expressing in the form of a fusion protein, and cleaving the glucagon derivative from the fusion protein using a protease or a compound which is capable of protein cleavage followed by separation. For this purpose, for example, an amino acid residue-encoding DNA sequence that can be cleaved by a protease such as Factor Xa or enterokinase, CNBr, or a compound such as hydroxylamine, may be inserted between the fusion partner and a polynucleotide encoding a glucagon derivative.

[0081] In a specific embodiment of the present invention, it was confirmed that the peptide of the present invention has a different pI compared to that of native glucagon (see Table 1). As a result, the peptide of the present invention has improved solubility and higher stability at a neutral pH. Accordingly, the peptide of the present invention can increase patient compliance when used as a hypoglycemic agent and is also suitable for combined administration of the peptide with other anti-obesity agents, and thus can be effectively used for the prevention and treatment of hypoglycemia and obesity.

[0082] In this regard, the peptide of the present invention can provide an attractive therapeutic selection regarding hypoglycemia, obesity, or associated diseases thereof.

[0083] For example, the peptide of the present invention is a major insulin response-controlling hormone, and can be effectively used for the treatment of severe hypoglycemia in diabetic patients.

[0084] Additionally, the peptide of the present invention may be used as a pharmaceutical medicament not only for preventing body weight increase, promotion of body weight decrease, reduction of overweight, and obesity including morbid obesity (e.g., by controlling appetite, ingestion, food intake, calorie intake, and/or energy consumption), but also for treating obesity-related inflammation, obesity-related gallbladder disease, and obesity-induced sleep apnea, but is not limited thereto, and may be used for treating the associated diseases or health conditions thereof.

[0085] The peptide of the present invention may also be used for treating metabolic syndrome other than obesity, i.e., obesity-related diseases such as impaired glucose tolerance, hypercholesterolemia, dyslipidemia, obesity, diabetes, hypertension, nonalcoholic steatohepatitis (nonalcoholic steatohepatitis, NASH), atherosclerosis caused by dyslipidemia, atherosclerosis, arteriosclerosis, coronary heart disease, stroke, hypoglycemia, etc. However, the effects of the peptide according to the present invention may be mediated entirely or partially by the body weight-related effects described above or may be independent of the same.

[0086] Examples of the compound or material having a therapeutic activity for metabolic syndrome to be included in the combined administration or the composition of the present invention may include an insulinotropic peptide in the form of a long-acting conjugate, to which a biocompatible material capable of increasing in vivo half-life of the insulinotropic peptide is linked.

[0087] Specifically, the insulinotropic peptide may be selected from the group consisting of GLP-1, exendin-3, exendin-4, an agonist thereof, a derivative thereof, a fragment thereof, a variant thereof, and a combination thereof.

[0088] More specifically, the insulinotropic peptide may be an insulinotropic peptide derivative in which the N-terminal histidine of the insulinotropic peptide is substituted with one selected from the group consisting of desamino-histidyl, N-dimethyl-histidyl, β -hydroxy imidazopropionyl, 4-imidazoacetyl, and β -carboxy imidazopropionyl, but is not limited thereto.

[0089] More specifically, the insulinotropic peptide may be selected from the group consisting of a native exendin-4; an exendin-4 derivative in which the N-terminal amine group of exendin-4 is deleted; an exendin-4 derivative in which the N-terminal amine group of exendin-4 is substituted with a hydroxyl group; an exendin-4 derivative in which the N-terminal amine group

of exendin-4 is modified with a dimethyl group; an exendin-4 derivative in which the α -carbon of the 1st amino acid of exendin-4, histidine, is deleted; an exendin-4 derivative in which the 12th amino acid of exendin-4, lysine, is substituted with serine, and an exendin-4 derivative in which the 12th amino acid of exendin-4, lysine, is substituted with arginine, but is not limited thereto.

[0090] In a "long-acting conjugate", the duration of efficacy of the conjugate increases compared to native glucagon or a glucagon derivative thereof, to which a biocompatible material is not linked.

[0091] Examples of the biocompatible material may include polyethylene glycol, fatty acid, cholesterol, albumin and a fragment thereof, an albumin-binding material, a polymer of repeating units of a particular amino acid sequence, an antibody, an antibody fragment, an FcRn-binding material, in vivo connective tissue or a derivative thereof, a nucleotide, fibronectin, transferrin, a saccharide, and a polymer, but are not limited thereto. For example, at least one amino acid side chain within the peptide of the present invention may be attached to the biocompatible material in order to increase *in vivo* solubility and/or half-life, and/or increase bioavailability thereof. These modifications are known to reduce the clearance of therapeutic proteins and peptides.

[0092] For the biocompatible polymer, soluble (amphipathic or hydrophilic), non-toxic, and pharmaceutically inert polymers are appropriate, and for example, they may include PEG, homopolymers or copolymers of PEG, monomethyl-substituted polymers (mPEG), and poly-amino acids such as poly-lysine, poly-aspartic acid, and polyglutamic acid, but are not limited thereto.

[0093] It is a known fact to a skilled person in the art that the thus-modified glucagon derivative would have a superior therapeutic effect compared to native glucagon. Accordingly, the variants of the glucagon derivative as described above also belong to the scope of the present invention.

[0094] In a more specific embodiment, the glucagon derivative and the insulinotropic peptide may be respectively linked to a biocompatible material by a linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, fatty acid, a polymer, a low molecular weight compound, a nucleotide, and a combination thereof, but is not limited thereto.

[0095] In an even more specific embodiment, the biocompatible material may be an FcRn-binding material, and the glucagon derivative, and the insulinotropic peptide may be respectively linked to a biocompatible material by a peptide linker or a non-peptide linker, but is not limited thereto.

[0096] As a specific example, the FcRn-binding material may be a polypeptide including an immunoglobulin Fc region.

[0097] As used herein, "non-peptide linker" includes a biocompatible polymer to which at least two repeating units are linked. The repeating units are linked with each other by a random covalent bond instead of a peptide bond. The non-peptide linker may be one constitution that establishes a moiety of a long-acting conjugate of the present invention.

[0098] As used herein, the term "non-peptide linker" may be used interchangeably with "non-peptide polymer".

[0099] Additionally, in a specific embodiment, the conjugate may be one in which the protein drug is covalently linked to the immunoglobulin Fc region by a non-peptide linker including a reactive group, which can be linked to the immunoglobulin Fc region and a protein drug on both ends of the conjugate.

[0100] Although not particularly limited, the non-peptide linker may be one selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, a polysaccharide, and a combination thereof. In a more specific embodiment, the non-peptide polymer may be polyethylene glycol, but is not limited thereto.

[0101] The non-peptide linker to be used in the present invention may be any polymer which has a resistance to in vivo proteases, without limitation. The molecular weight of the non-peptide polymer may be in the range of 1 kDa to 100 kDa, and specifically, 1 kDa to 20 kDa, but is not limited thereto. Additionally, the non-peptide linker, which is linked to the polypeptide including the immunoglobulin Fc region, may include not only a single kind of a polymer but also a combination of different kinds of polymers.

[0102] In a specific embodiment, both ends of the non-peptide linker may be respectively linked to an amine group or a thiol group of a peptide, which comprises the amino acid sequence of SEQ ID NO: 20 or 37 or an insulinotropic peptide, and a biocompatible material.

[0103] Specifically, the non-peptide polymer may include a reactive group on both ends, respectively, which can be linked to an immunoglobulin Fc fragment and, a glucagon derivative which comprises the amino acid sequence of SWQ ID NO: 30 or 37 or an insulinotropic peptide; and specifically, a reactive group which can be linked to an amine group of N-terminus or lysine, or a thiol group of cysteine of the glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37 or the insulinotropic peptide, or the immunoglobulin Fc fragment.

[0104] Additionally, the reactive group of the non-peptide polymer that can be linked to the

which comprises the amino acid sequence of SEQ ID NO: 20 or 37 immunoglobulin Fc region, the glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37, and the insulinotropic peptide may be selected from the group consisting of an aldehyde group, a maleimide group, and a succinimide derivative, but is not limited thereto.

[0105] In the above, examples of the aldehyde group may include a propionaldehyde group or a butyraldehyde group, but are not limited thereto.

[0106] In the above, as a succinimide derivative, succinimidyl valerate, succinimidyl methylbutanoate, succinimidyl methylpropionate, succinimidyl butanoate, succinimidyl propionate, N-hydroxysuccinimide, hydroxy succinimidyl, succinimidyl carboxymethyl, or succinimidyl carbonate may be used, but is not limited thereto.

[0107] Additionally, the final product produced through reductive alkylation via an aldehyde bond is more stable than that linked by an amide bond. The aldehyde reactive group selectively reacts with a N-terminus at a low pH condition while it can form a covalent bond with a lysine residue at high pH, e.g., pH 9.0.

[0108] The reactive groups at both ends of the non-peptide linker may be the same as or different from each other, for example, a maleimide reactive group may be provided at one end and an aldehyde group, a propionaldehyde group, or a butyraldehyde group may be provided at the other end. However, if an immunoglobulin Fc region and a glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37 or an insulinotropic peptide can be conjugated at each end of the non-peptide linker, it is not particularly limited.

[0109] For example, the non-peptide polymer may possess a maleimide group at one end and an aldehyde group, a propionaldehyde group, or a butyraldehyde group at the other end.

[0110] When a polyethylene glycol having a reactive hydroxy group at both ends thereof is used as the non-peptide polymer, the hydroxy group may be activated to various reactive groups by known chemical reactions, or a polyethylene glycol having a commercially available modified reactive group may be used so as to prepare the long-acting protein conjugate of the present invention.

[0111] In a specific embodiment, the non-peptide polymer may be one which can be linked to a cysteine residue of a glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37, and more specifically, to the -SH group of cysteine, but is not limited thereto.

[0112] When maleimide-PEG-aldehyde is used, the maleimide group may be linked to the -SH group of the glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37 by a thioether bond and the aldehyde group may be linked to the -NH₂ of the immunoglobulin Fc through reductive alkylation, but is not limited thereto.

[0113] "immunoglobulin Fc region" refers to a region including the heavy chain constant region

2 (CH2) and/or the heavy chain constant region 3 (CH3), excluding the heavy chain and light chain variable regions of an immunoglobulin. The immunoglobulin Fc region may be one constitution that establishes a moiety of a protein conjugate of the present invention.

[0114] The immunoglobulin Fc region may include a hinge region in the heavy chain constant region, but is not limited thereto. Additionally, the immunoglobulin Fc region may be an extended Fc region including a part or the entirety of the heavy chain constant region 1 (CH1) and/or the light chain constant region 1 (CL1), excluding the heavy chain and the light chain variable regions of the immunoglobulin, as long as the immunoglobulin Fc region has an effect substantially the same as or improved compared to the native type. Additionally, the immunoglobulin Fc region may be a region in which a fairly long part of the amino acid sequence corresponding to CH2 and/or CH3 is removed.

[0115] For example, the immunoglobulin Fc region may be 1) a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain; 2) a CH1 domain and a CH2 domain; 3) a CH1 domain and a CH3 domain; 4) a CH2 domain and a CH3 domain; 5) a combination between one or two or more domains among a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain and an immunoglobulin hinge region (or a part of the hinge region); and 6) a dimer between each domain of the heavy chain constant region and the light chain constant region, but is not limited thereto.

[0116] Additionally, in a specific embodiment, the immunoglobulin Fc region may be in a dimeric form, and one molecule of a glucagon derivative or insulintropic peptide may be covalently linked to a Fc region in a dimeric form, and in particular, the immunoglobulin Fc and the glucagon derivative which comprises the amino acid sequence of SEQ ID NO: 20 or 37 or the insulintropic peptide may be interlinked by a non-peptide polymer. Furthermore, two molecules of the glucagon derivative or insulintropic peptide may be possibly conjugated in a symmetrical manner to a single Fc region in a dimeric form. In particular, the immunoglobulin Fc and the glucagon derivative or the insulintropic peptide may be interlinked by a non-peptide linker, but are not limited to the embodiment described above.

[0117] Additionally, the immunoglobulin Fc region not only includes a native amino acid sequence but also a sequence derivative thereof. An amino acid sequence derivative refers to an amino acid sequence which has a difference in at least one amino acid residue due to deletion, insertion, non-conservative or conservative substitution, or a combination thereof.

[0118] For example, the amino acid residues at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331, which are known to be in the binding of an immunoglobulin Fc, may be used as suitable sites for modification.

[0119] Additionally, other various derivatives are possible, including one that has a deletion of a region capable of forming a disulfide bond, or a deletion of some amino acid residues at the N-terminus of native Fc or an addition of a methionine residue at the N-terminus of native Fc. Further, to remove effector functions, a deletion may occur in a complement-binding site, such

as a C1q-binding site and an antibody dependent cell mediated cytotoxicity (ADCC) site. Techniques of preparing such sequence derivatives of the immunoglobulin Fc region are disclosed in International Patent Publication Nos. WO 97/34631, WO 96/32478, etc.

[0120] Amino acid exchanges in proteins and peptides, which do not generally alter the activity of the proteins or peptides, are known in the art (H. Neurath, R. L. Hill, *The Proteins*, Academic Press, New York, 1979). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly, in both directions. In addition, the Fc region may, if necessary, be modified by phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, etc.

[0121] The above-described Fc derivatives show biological activity identical to that of the Fc region described above and have improved structural stability against heat, pH, etc.

[0122] Further, the immunoglobulin Fc region may be obtained from native forms isolated *in vivo* from humans or animals such as cows, goats, pigs, mice, rabbits, hamsters, rats, guinea pigs, etc., or may be recombinants or derivatives thereof, obtained from transformed animal cells or microorganisms. Herein, the Fc region may be obtained from a native immunoglobulin by isolating a whole immunoglobulin from a living human or animal body and treating the isolated immunoglobulin with protease. When the whole immunoglobulin is treated with papain, it is cleaved into Fab and Fc regions, whereas when the whole immunoglobulin is treated with pepsin, it is cleaved into pF'c and F(ab)₂ fragments. Fc or pF'c can be isolated using size exclusion chromatography, etc. In a more specific embodiment, a human-derived Fc region is a recombinant immunoglobulin Fc region obtained from a microorganism.

[0123] In addition, the immunoglobulin Fc region may have natural glycans, increased or decreased glycans compared to the natural type, or be in a deglycosylated form. The increase, decrease, or removal of the glycans of the immunoglobulin Fc may be achieved by conventional methods such as a chemical method, an enzymatic method, and a genetic engineering method using a microorganism. The immunoglobulin Fc region obtained by removal of glycans from the Fc region shows a significant decrease in binding affinity to the C1q part and a decrease or loss in antibody-dependent cytotoxicity or complement-dependent cytotoxicity, and thus it does not induce unnecessary immune responses *in vivo*. In this regard, an immunoglobulin Fc region in a deglycosylated or aglycosylated immunoglobulin Fc region may be a more suitable form to meet the original object of the present invention as a drug carrier.

[0124] As used herein, the term "deglycosylation" refers to enzymatically removing sugar moieties from an Fc region, and the term "aglycosylation" refers to an unglycosylated Fc region produced in prokaryotes, more specifically, *E. coli*.

[0125] Meanwhile, the immunoglobulin Fc region may be derived from humans or other animals including cows, goats, pigs, mice, rabbits, hamsters, rats, and guinea pigs. In a more

specific embodiment, it is derived from humans.

[0126] In addition, the immunoglobulin (Ig) Fc region may be derived from IgG, IgA, IgD, IgE, IgM, or a combination or hybrid thereof. In a more specific embodiment, it is derived from IgG or IgM, which are among the most abundant proteins in human blood, and in an even more specific embodiment, it is derived from IgG, which is known to enhance the half-lives of ligand-binding proteins. In a yet even more specific embodiment, the immunoglobulin Fc region is an IgG4 Fc region, and in the most specific embodiment, the IgG4 Fc region is an aglycosylated Fc region derived from human IgG4, but is not limited thereto.

[0127] In particular, as used herein, the term "combination" means that polypeptides encoding single-chain immunoglobulin Fc regions of the same origin are linked to a single-chain polypeptide of a different origin to form a dimer or multimer. That is, a dimer or multimer may be formed from two or more fragments selected from the group consisting of IgG Fc, IgA Fc, IgM Fc, IgD Fc, and IgE Fc fragments.

[0128] The composition of the present invention can be used for preventing or treating hypoglycemia or metabolic syndromes as set out in the claims.

[0129] As used herein, the term "prevention" refers to all kinds of actions associated with the inhibition or delay of the occurrence of hypoglycemia or metabolic syndrome by the administration of the peptide or the composition, and the term "treatment" refers to all kinds of actions associated with the improvement or advantageous changes in symptoms of hypoglycemia or metabolic syndrome by the administration of the peptide or the composition.

[0130] As used herein, the term "administration" refers to an introduction of a particular material to a patient by an appropriate manner. The composition may be administered by a general route that enables the delivery of the composition to a target tissue *in vivo*, for example, intraperitoneal, intravenous, intramuscular, subcutaneous, intradermal, oral, topical, intranasal, intrapulmonary, and intrarectal administration, but is not particularly limited thereto.

[0131] As used herein, the term "metabolic syndrome" refers to a symptom of a single or complex occurrence of various diseases due to chronic metabolic disorder, and in particular, examples of metabolic syndrome may include impaired glucose tolerance, hypercholesterolemia, dyslipidemia, obesity, diabetes, hypertension, nonalcoholic steatohepatitis (NASH), atherosclerosis caused by dyslipidemia, atherosclerosis, arteriosclerosis, coronary heart disease, stroke, etc., but are not limited thereto.

[0132] As used herein, the term "obesity" refers to a medical condition with excess body fat in the body, and a person having a body mass index (BMI; body mass (kg) divided by the square of the body height (m)) of 25 or higher is diagnosed as having obesity. Obesity generally occurs due to a long-term energy imbalance in which energy intake exceeds energy expenditure. Obesity is a metabolic disease that affects the entire body, which increases the risk of diabetes, hyperlipidemia, sexual dysfunction, arthritis, and cardiovascular disease, and

in some cases, it is also associated with the occurrence of cancers.

[0133] Diabetes may represent "hypoglycemia" as an acute symptom.

[0134] As used herein, the term "hypoglycemia" refers to an acute symptom of diabetes, in which blood glucose levels are lower than those of normal people, and in general, refers to a state when the blood glucose levels are 50 mg/dL or less. Hypoglycemia is frequently caused when a person who takes an oral hypoglycemic agent or insulin has eaten less than usual or has performed activities or exercised more than usual. In addition, hypoglycemia may occur due to the use of glucose level-lowering drugs, severe physical diseases, deficiency in hormones such as adrenocortical hormones and glucagon, tumor in insulin-producing pancreas, autoimmune insulin syndrome, gas-trectomy patients, hereditary carbohydrate metabolism disorder, etc.

[0135] Symptoms of hypoglycemia include weakness, trembling, pale skin, cold sweats, dizziness, excitement, anxiety, pounding heart, empty stomach, headache, fatigue, etc. In the case of persistent hypoglycemia, it may lead to convulsion or seizure, and may cause shock and thus fainting.

[0136] The pharmaceutical composition of the present invention may contain a pharmaceutically acceptable carrier, excipient, or diluent. As used herein, the term "pharmaceutically acceptable" refers to the properties of having a sufficient amount to exhibit a therapeutic effect and not causing adverse effects, and may be easily determined by a skilled person in the art based on the factors well known in the medical field, such as the kind of disease, age, body weight, health status, sex, drug sensitivity of a patient, administration route, administration method, administration frequency, duration of treatment, a drug to be mixed or administered simultaneously in combination, etc.

[0137] The pharmaceutical composition of the present invention containing the peptide of the present invention may further contain a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may include, for oral administration, a binder, a glidant, a disintegrant, an excipient, a solubilizing agent, a dispersant, a stabilizing agent, a suspending agent, a coloring agent, a flavoring agent, etc.; for injections, a buffering agent, a preserving agent, an analgesic, a solubilizing agent, an isotonic agent, a stabilizing agent, etc., which may be combined to be used; and for topical administrations, a base, an excipient, a lubricant, a preserving agent, etc., although it is not limited thereto.

[0138] The formulation type of the composition according to the present invention may be prepared variously by combining with a pharmaceutically acceptable carrier as described above. For example, for oral administration, the composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups, wafers, etc. For injections, the composition may be formulated into single-dose ampoules or multidose containers. The composition may be also formulated into solutions, suspensions, tablets, capsules, and sustained-release formulations.

[0139] Meanwhile, examples of suitable carriers, excipients, and diluents may include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate, mineral oil, etc. Additionally, the composition may further contain a filler, an anti-coagulant, a lubricant, a humectant, a flavoring agent, a preservative, etc.

[0140] Additionally, the pharmaceutical composition of the present invention may be prepared in any formulation type selected from the group consisting of tablets, pills, powders, granules, capsules, suspensions, liquid medicine for internal use, emulsions, syrups, sterile injection solutions, non-aqueous solvents, lyophilized formulations, and suppositories.

[0141] Additionally, the composition may be formulated into a single dosage form suitable for the patient's body, and preferably is formulated into a preparation useful for peptide drugs according to the typical method used in the pharmaceutical field to be administered by an oral or parenteral route, such as through skin, intravenously, intramuscularly, intra-arterially, intramedullarily, intrathecally, intraventricularly, pulmonarily, transdermally, subcutaneously, intraperitoneally, intranasally, intragastrically, topically, sublingually, vaginally, or rectally, but is not limited thereto.

[0142] Additionally, the peptide may be used by blending with various pharmaceutically acceptable carriers such as physiological saline or organic solvents. In order to increase the stability or absorptivity, carbohydrates such as glucose, sucrose, or dextrans; antioxidants such as ascorbic acid or glutathione; chelating agents; low molecular weight proteins; or other stabilizers may be used.

[0143] The administration dose and frequency of the pharmaceutical composition of the present invention are determined by the type of active ingredient(s), along with various factors, such as the disease to be treated, administration route, patient's age, sex, and body weight, and severity of the disease.

[0144] The total effective dose of the composition of the present invention may be administered to a patient in a single dose, or may be administered for a long period of time in multiple doses according to a fractionated treatment protocol. In the pharmaceutical composition of the present invention, the content of active ingredient(s) may vary depending on the disease severity. Specifically, the preferable total daily dose of the peptide of the present invention may be approximately 0.0001 μg to 500 mg per 1 kg of body weight of a patient. However, the effective dose of the peptide is determined considering various factors including patient's age, body weight, health conditions, sex, disease severity, diet, and excretion rate, in addition to administration route and treatment frequency of the pharmaceutical composition. In this regard, those skilled in the art may easily determine the effective dose suitable for the particular use of the pharmaceutical composition of the present invention. The pharmaceutical composition according to the present invention is not particularly limited to the formulation and

administration route and mode, as long as it shows the effects of the present invention.

[0145] The pharmaceutical composition of the present invention shows excellent *in vivo* duration of efficacy and titer, and thus the number and frequency of administration of the pharmaceutical preparation of the present invention can be significantly reduced.

[0146] In particular, since the pharmaceutical composition of the present invention contains, as an active ingredient, a glucagon derivative comprising the amino acid sequence of SEQ ID NO: 20 or 30 having an altered pI different from that of native glucagon, it shows improved solubility and high stability according to the pH of a given solution, and thus the pharmaceutical composition of the present invention can be effectively used in the preparation of a stable glucagon formulation for treating hypoglycemia or obesity as set out in the claims.

[0147] In another aspect, the present invention provides a novel glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37.

[0148] the C-terminus of the peptide including the amino acid sequence of SEQ ID NO: 37 may be amidated, but is not limited thereto.

[0149] Additionally, the peptide may be a glucagon derivative capable of activating a glucagon receptor, but is not limited thereto.

[0150] In still another aspect, the present invention provides an isolated polynucleotide encoding the glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37, a vector including the polynucleotide, and an isolated cell including the polynucleotide or the vector.

[0151] Additionally, the isolated polynucleotide encoding the glucagon derivative includes within the scope of the present invention a polynucleotide sequence having a homology of 75% or higher, specifically 85% or higher, more specifically 90% or higher, and even more specifically 95% or higher, to the corresponding sequence.

[0152] As used herein, the term "homology" indicates sequence similarity with a wild-type amino acid sequence or wild-type nucleotide sequence, and the homology comparison may be done with the naked eye or using a commercially available comparison program. Using a commercially available computer program, the homology between two or more sequences may be expressed as a percentage (%), and the homology (%) between adjacent sequences may be calculated.

[0153] As used herein, the term "recombinant vector" refers to a DNA construct including the sequence of a polynucleotide encoding a target peptide, e.g., a glucagon derivative, which is operably linked to an appropriate regulatory sequence to enable the expression of the target peptide, e.g., a glucagon derivative, in a host cell.

[0154] The regulatory sequence includes a promoter capable of initiating transcription, any operator sequence for the regulation of the transcription, a sequence encoding an appropriate mRNA ribosome-binding domain, and a sequence regulating the termination of transcription and translation. The recombinant vector, after being transformed into a suitable host cell, may be replicated or function irrespective of the host genome, or may be integrated into the host genome itself.

[0155] The recombinant vector used in the present invention may not be particularly limited as long as the vector is replicable in the host cell, and it may be constructed using any vector known in the art. Examples of the vector conventionally used may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. The vectors to be used in the present invention may be any expression vector known in the art.

[0156] The recombinant vector is used for the transformation of a host cell for producing glucagon derivatives of the present invention. Additionally, these transformed cells, as a part of the present invention, may be used for the amplification of nucleic acid fragments and vectors, or may be cultured cells or cell lines used in the recombinant production of glucagon derivatives of the present invention.

[0157] As used herein, the term "transformation" refers to a process of introducing a recombinant vector including a polynucleotide encoding a target protein into a host cell, thereby enabling the expression of the protein encoded by the polynucleotide in the host cell. For the transformed polynucleotide, it does not matter whether it is inserted into the chromosome of a host cell and located thereon or located outside of the chromosome, as long as it can be expressed in the host cell, and both cases are included.

[0158] Additionally, the polynucleotide includes DNA and RNA which encode the target protein. The polynucleotide may be inserted in any form insofar as it can be introduced into a host cell and expressed therein. For example, the polynucleotide may be introduced into a host cell in the form of an expression cassette, which is a gene construct including all the essential elements required for self-expression. The expression cassette may conventionally include a promoter operably linked to the polynucleotide, a transcription termination signal, a ribosome-binding domain, and a translation termination signal. The expression cassette may be in the form of an expression vector capable of self-replication. Additionally, the polynucleotide may be introduced into a host cell as it is and operably linked to a sequence essential for its expression in the host cell, but is not limited thereto.

[0159] Additionally, as used herein, the term "operably linked" refers to a functional connection between a promoter sequence, which initiates and mediates the transcription of the polynucleotide encoding the target peptide of the present invention, and the above gene sequence.

[0160] An appropriate host to be used in the present invention may not be particularly limited as long as it can express the polynucleotide of the present invention. Examples of the

appropriate host may include bacteria belonging to the genus *Escherichia* such as *E. coli*; bacteria belonging to the genus *Bacillus* such as *Bacillus subtilis*; bacteria belonging to the genus *Pseudomonas* such as *Pseudomonas putida*; yeasts such as *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*; insect cells such as *Spodoptera frugiperda* (Sf9), and animal cells such as CHO, COS, and BSC.

[0161] In still another aspect, the present invention provides an isolated conjugate in which a glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37 and a biocompatible material which is capable of increasing in vivo half-life are linked. The conjugate may be a long-acting conjugate.

[0162] Regarding the glucagon derivative, the biocompatible material, and the constitution of the conjugate, all those described above are applied.

[0163] Specifically, the biocompatible material may be selected from the group consisting of polyethylene glycol, fatty acid, cholesterol, albumin and a fragment thereof, an albumin-binding material, a polymer of repeating units of a particular amino acid sequence, an antibody, an antibody fragment, an FcRn-binding material, in vivo connective tissue or a derivative thereof, a nucleotide, fibronectin, transferrin, saccharide, and a polymer, but is not limited thereto.

[0164] Additionally, the isolated peptide may be linked to a biocompatible material by a linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, fatty acid, a polymer, a low molecular weight compound, a nucleotide, and a combination thereof, but is not limited thereto.

[0165] Additionally, the biocompatible material may be an FcRn-binding material, and the isolated peptide may be linked to a biocompatible material by a peptide linker or a non-peptide linker selected from the group consisting of polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, a polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymer, chitin, hyaluronic acid, and a combination thereof, but is not limited thereto.

[0166] Additionally, the FcRn-binding material may be a polypeptide including the immunoglobulin Fc region, but is not limited thereto.

[0167] In still another aspect, the present invention provides a composition containing the glucagon derivative comprising the amino acid sequence of SEQ ID NO: 37 or the isolated conjugate.

[0168] The glucagon derivative and the isolated conjugate are the same as explained above.

[0169] Specifically, the composition may be a pharmaceutical composition for treating or preventing hypoglycemia or metabolic syndrome, but is not limited thereto. The pharmaceutical composition is the same as described above.

Mode for the Invention

[0170] Hereinafter, the present invention will be described in more detail with reference to the following examples and experimental examples. However, the following examples and experimental examples are provided for illustrative purposes only, and the scope of the present invention should not be limited thereto in any manner.

Example 1: Production of cell line showing cAMP response to glucagon

[0171] PCR was performed using a region corresponding to Open Reading Frame (ORF) in the cDNA (OriGene Technologies, Inc., USA) of human glucagon receptor gene as a template along with the following forward and reverse primers (SEQ ID NOS: 47 and 48, respectively), which include each of the *EcoRI* and *XhoI* restriction sites.

[0172] In particular, PCR was performed for a total of 30 cycles under the following conditions: 95°C denaturation for 60 sec, annealing at 55°C for 60 sec, and polymerization at 68°C for 30 sec. The amplified PCR products were subjected to a 1.0% agarose gel electrophoresis and a 450 bp band was obtained by elution.

[0173] Forward primer (SEQ ID NO: 47):
5'-CAGCGACACCGACCGTCCCCCGTACTTAAGGCC-3'

[0174] Reverse primer (SEQ ID NO: 48):
5'-CTAACCGACTCTCGGGGAAGACTGAGCTCGCC-3'

[0175] The PCR product was cloned into a known animal cell expression vector, x0GC/dhfr, to prepare a recombinant vector x0GC/GCGR.

[0176] CHO DG44 cell line cultured in DMEM/F12 (10% FBS) medium was transfected with the recombinant vector x0GC/GCGR using Lipofectamine[®], and cultured in a selection medium containing G418 (1 mg/mL) and methotrexate (10 nM). Single clone cell lines were selected therefrom by a limit dilution technique, and a cell line showing excellent cAMP response to glucagon in a concentration-dependent manner was finally selected therefrom.

Example 2: Synthesis of glucagon derivative

[0177] In order to prepare glucagon derivatives with improved physical properties, the amino acid sequence of native glucagon of SEQ ID NO: 1 was substituted with amino acid residues having positive and negative charges, and thereby glucagon derivatives were synthesized as shown in Table 1 below. The relative *in vitro* activities described below were measured by the method described in Example 4.

[Table 1]

Amino acid sequences of native glucagon and glucagon derivatives				
SEQ ID NO	Peptide Sequence	Ring Formation	pI	<i>In vitro</i> Activity (Relative Activity of SEQ ID NO: 1, %)
SEQ ID NO: 1	HSQGTFTSDYSKYLD ^{SRRAQD} FVQWLMNT	-	6.8	100
SEQ ID NO: 2	HSQGTFTSDYSKYLD ^{CDRAQ} DFVQWLMNT	-	4.56	0.6
SEQ ID NO: 3	HSQGTFTSDYSKYLD ^{CERAQ} DFVQWLMNT	-	4.66	6.1
SEQ ID NO: 4	HSQGTFTSDYSKYLD ^{SCDAQ} DFVQWLMNT	-	4.13	< 0.1
SEQ ID NO: 5	HSQGTFTSDYSKYLD ^{SCEAQD} FVQWLMNT	-	4.22	0.3
SEQ ID NO: 6	HSQGTFTSDYSKYLD ^{SCEADD} FVQWLMNT	-	4.03	< 0.1
SEQ ID NO: 7	YSQGTFTSDYSKYLD ^{SCEADD} FVQWLMNT	-	3.71	< 0.1
SEQ ID NO: 8	YXQGTFTSDYSKYLD ^{SCDAQ} DFVQWLINT	-	3.77	< 0.1

Amino acid sequences of native glucagon and glucagon derivatives				
SEQ ID NO	Peptide Sequence	Ring Formation	pI	In vitro Activity (Relative Activity of SEQ ID NO: 1, %)
SEQ ID NO: 9	YXQGTFTSDYSKYLDSCDAQ DFVVWLINT	-	3.77	< 0.1
SEQ ID NO: 10	YXQGTFTSDYSKYLDSCDAD DFVVWLINT	-	3.66	< 0.1
SEQ ID NO: 11	YXQGTFTSDYSKYLDEKCAK EFVQWLMNT	-	4.78	4.6
SEQ ID NO: 12	YXQGTFTSDYSKYLDEKRAK EFVQWLMNTC	ring formed	6.20	56.3
SEQ ID NO: 13	YXQGTFTSDYSCYLDSRRAQ DFVQWLMNT	-	4.43	5.2
SEQ ID NO: 14	YXQGTFTSDYSKYLDCKRAK EFVQWLMNT	-	8.12	18.1
SEQ ID NO: 15	YXQGTFTSDYSKYLCEKRAQ DFVVWLMNT	-	6.11	1.1
SEQ ID NO: 16	YXQGTFTSDYSKYLDCRRAQ VFVQWLMRT	-	9.11	4.2
SEQ ID NO: 17	YXQGTFTSDYSKYLDCVRAQ DFVQWLMRT	-	6.03	23.2
SEQ ID NO: 18	YXQGTFTSDYSKYLDSRRAC DFRLWLMNT	-	8.15	< 0.1

Amino acid sequences of native glucagon and glucagon derivatives				
SEQ ID NO	Peptide Sequence	Ring Formation	pI	In vitro Activity (Relative Activity of SEQ ID NO: 1, %)
SEQ ID NO: 19	YXQGTFTSDYSKYLC <u>E</u> KRAK EFVQWLMNT	ring formed	8.12	12.1
SEQ ID NO: 20	YXQGTFTSDYSKYLD <u>E</u> CRAK EFVQWLMNT	ring formed	4.78	299.7
SEQ ID NO: 21	YXQGTFTSDYSKYLD <u>E</u> KCAK EFVQWLMNT	ring formed	4.78	57.8
SEQ ID NO: 22	YXQGTFTSDYSKYLD <u>E</u> KRCK EFVQWLMNT	ring formed	6.20	147.8
SEQ ID NO: 23	YXQGTFTSDYSKYCD <u>E</u> KRAK EFVQWLMNT	ring formed	6.20	76.8
SEQ ID NO: 24	YXQGTFTSDYSKCLD <u>E</u> KRAK EFVQWLMNT	ring formed	6.21	58.0
SEQ ID NO: 25	YXQGTFTSDYSKYLD <u>E</u> KRAK CFVQWLMNT	ring formed	8.12	46.9
SEQ ID NO: 26	WXQGTFTSDYSKYLD <u>E</u> CRAK DFVQWLMNT	ring formed	4.68	1.0
SEQ ID NO: 27	YXQGTFTSDYSKYLD <u>E</u> CRAK DFVQWLMNT	ring formed	4.68	93.6
SEQ ID NO: 28	WXQGTFTSDYSKYLD <u>E</u> CRAK DFVQWLMNT	ring formed	4.68	< 0.1
SEQ ID NO: 29	YXQGTFTSDYSKCLD <u>E</u> RAK	ring formed	6.15	61.3

Amino acid sequences of native glucagon and glucagon derivatives				
SEQ ID NO	Peptide Sequence	Ring Formation	pI	In vitro Activity (Relative Activity of SEQ ID NO: 1, %)
	DFVQWLMNT			
SEQ ID NO: 30	WXQGTFTSDYSKCLDERRAK DFVQWLMNT	ring formed	4.44	0.3
SEQ ID NO: 31	YXQGTFTSDYSKYLDCRAK EFVQWLMNT	ring formed	8.12	6.3
SEQ ID NO: 32	-SQGTFTSDYSKYLDECRAKE FVQWLMNT	ring formed	4.78	0.7
SEQ ID NO: 33	YXQGTFTSDYSKYLDSRRAQ DFVQWLMNT	-	6.04	108.2
SEQ ID NO: 34	WXQGTFTSDYSKYCDERRAK EFVQWLMNT	ring formed	6.21	0.2
SEQ ID NO: 35	YXQGTFTSDYSKYCDERRAK EFVQWLMNT	ring formed	6.2	17.7
SEQ ID NO: 36	YXQGTFTSDCSKYLDERRAK EFVQWLMNT	ring formed	6.21	9.9
SEQ ID NO: 37	YXQGTFTSDYSKYLDERRAK EFVQWLMNTC	ring formed	6.21	225.5
SEQ ID NO: 38	YXQGTFCSDYSKYLDERRAK EFVQWLMNT	ring formed	6.15	167.3
SEQ ID NO: 39	YXQGTFSVDCSKYLDERRAK DFVQWLMNT	ring formed	6.15	3.7

Amino acid sequences of native glucagon and glucagon derivatives				
SEQ ID NO	Peptide Sequence	Ring Formation	pI	In vitro Activity (Relative Activity of SEQ ID NO: 1, %)
SEQ ID NO: 40	YXQGTfVSDYSKYLD <u>ERRAK</u> DFVQWLMNTC	ring formed	6.15	40.8
SEQ ID NO: 41	YXQGTfCSDYSKYLD <u>ERRAK</u> DFVQWLMNT	ring formed	6.03	45.2
SEQ ID NO: 42	YXQGTfCSDYSKYLDSRRAQ DFVQWLMNT	-	6.03	37.9
SEQ ID NO: 43	YXQGTfTSDCSKYLDSRRAQ DFVQWLMNT	-	6.03	1.6
SEQ ID NO: 44	YXQGTfTSDYSKYLDSRRAQ DFVQWLMNTC	-	6.21	75.4

[0178] In the amino acids sequences described in Table 1, the amino acid represented by X represents a non-native amino acid, aminoisobutyric acid(Aib), the underlined amino acid residues represent formation of a ring, and "-" in the amino acid sequence indicates that no amino acid residue is present on the corresponding position.

Example 3: Measurement of pI of glucagon derivatives

[0179] In order to measure the improved physical properties of glucagon derivatives synthesized in Example 2, pI values were calculated based on the amino acid sequences using the pI/Mw tool (http://expasy.org/tools/pi_tool.html; Gasteiger *et al.*, 2003) in the ExPASy server.

[0180] As shown in Table 1 above, while the native glucagon of SEQ ID NO: 1 had a pI of 6.8, some glucagon derivatives showed pI values in the range of from about 4 to about 6. derivatives having pI values lower or more than that of native glucagon can exhibit improved solubility and higher stability at a neutral pH condition compared to native glucagon.

[0181] Accordingly, when the glucagon derivatives are used as a therapeutic agent for treating hypoglycemia, they can improve patient compliance, and are also suitable for administration in combination with other anti-obesity agents or anti-diabetes agents, and thus the glucagon derivatives of the present invention can be effectively used as a therapeutic agent for treating hypoglycemia and metabolic syndromes including obesity, diabetes, nonalcoholic steatohepatitis (NASH), dyslipidemia, and coronary heart disease.

Example 4: Measurement of cAMP activity of glucagon derivatives

[0182] The activities of the glucagon derivatives synthesized in Example 2 were measured in cell lines having the human glucagon receptors produced in Example 1. Specifically, the transfected cell line was subcultured 3 to 4 times a week, aliquoted into a 384-well plate in an amount of 6×10^3 cell lines/well, and cultured for 24 hours. Native glucagon and glucagon derivatives were suspended in Hank's balanced salt solution (HBSS) buffer containing 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX), 0.1% bovine serum albumin (BSA), and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with the culture cells, at concentrations of 200 nM and 1600 nM, respectively, continuously subjected into a 4-fold dilution 10 times, applied to a cAMP assay kit (LANCE cAMP 384 kit, PerkinElmer), and added to the cultured cells, and their fluorescence value was measured. Upon measurement, the highest fluorescence value was set at 100% and then EC_{50} values of the glucagon derivative were calculated based on the same and compared with that of native glucagon, respectively. The results are shown in Table 1 above.

[0183] Example 5: Preparation of a conjugate including a glucagon derivative and an immunoglobulin Fc (SEO ID NO: 12 or 20-immunoglobulin Fc region conjugate)

[0184] For the pegylation of a 10kDa PEG having a maleimide group and an aldehyde group, respectively, at both ends (named as "maleimide-PEG-aldehyde", 10 kDa, NOF, Japan) into the cysteine residue of a glucagon derivative (SEQ ID NOS: 12 and 20), the glucagon derivatives and maleimide-PEG-aldehyde were reacted at a molar ratio of 1 : 1 to 5, at a protein concentration of 3 mg/mL to 10 mg/mL at low temperature for 1 to 3 hours. In particular, the reaction was conducted in an environment in which 20% to 60% isopropanol was added. Upon completion of the reaction, the reactants were applied to SP sepharose HP (GE healthcare, USA) to purify the glucagon derivatives mono-pegylated on cysteine.

[0185] Then, the purified mono-pegylated glucagon derivatives and an immunoglobulin Fc were reacted at a molar ratio of 1 : 2 to 10, at a protein concentration of 10 mg/mL to 50 mg/mL at 4°C to 8°C for 12 hours to 18 hours. The reaction was conducted in an environment in which sodium cyanoborohydride ($NaCNBH_3$) and 10% to 20% isopropanol were added to 100mM calcium phosphate buffer (pH 6.0). Upon completion of the reaction, the reactants were applied to the Butyl sepharose FF purification column (GE healthcare, USA) and Source ISO purification column (GE healthcare, USA) to purify the conjugate including the glucagon

derivatives and the immunoglobulin Fc.

[0186] After preparation, the purity analyzed by reverse phase chromatography, size exclusion chromatography, and ion exchange chromatography was shown to be 95% or higher.

[0187] In particular, the conjugate in which the glucagon derivative of SEQ ID NO: 12 and an immunoglobulin Fc were linked by PEG was named as "the conjugate including the glucagon derivative of SEQ ID NO: 12 and an immunoglobulin Fc" or "a long-acting derivative of SEQ ID NO: 12", and they can be interchangeably used.

[0188] In particular, the conjugate in which the glucagon derivative of SEQ ID NO: 20 and an immunoglobulin Fc were linked by PEG was named as "a conjugate including the glucagon derivative of SEQ ID NO: 20 and an immunoglobulin Fc" or "a long-acting derivative of SEQ ID NO: 20", and they can be interchangeably used.

[0189] Example 6: Preparation of a conjugate including an exendin-4 derivative and an immunoglobulin Fc

[0190] A 3.4 kDa PEG having a propionaldehyde group at both ends, i.e., 3.4k PropionALD (2) PEG, was reacted with the Lys of CA exendin-4 using imidazo-acetyl exendin-4 where the alpha carbon of N-terminal histidine was deleted (CA exendin-4, AP, USA), and then a coupling was conducted based on the isomer peak at the rearmost part (Lys27) between the two Lys peaks, which is quite reactive and clearly distinguished from the N-terminal isomer.

[0191] A peptide and an immunoglobulin Fc were reacted at a molar ratio of 1 : 8, at the total protein concentration of 60 mg/mL at 4°C for 20 hours. The reactant was 100 mM K-P (pH 6.0) and 20 mM SCB, a reducing agent, was added. The coupling reactants were purified by passing through with two purification columns. First, a large amount of immunoglobulin Fc not involved in the coupling reaction was removed using the SOURCE Q (XK-16mL, Amersham Biosciences). Upon application of a salt gradient using 1 M NaCl at 20 mM Tris (pH 7.5) results in the immediate elution of the immunoglobulin Fc, which has a relatively weak binding affinity, followed immediately by the elution of exendin-4-immunoglobulin Fc. The immunoglobulin Fc is removed to some extent by the primary purification, however, complete separation was not achieved by ion exchange column because of the small difference in binding affinity between the immunoglobulin Fc and the exendin-4-immunoglobulin Fc. Accordingly, secondary purification was performed using the hydrophobicity of the two different materials. The sample, which passed through the primary purification, was bound to the SOURCE ISO (HR16 mL, Amersham Biosciences) using 20 mM Tris (pH 7.5) and 1.5 M ammonium sulfate, and was then eluted while the concentration of ammonium sulfate was gradually lowered. As a result, the immunoglobulin Fc, which has a weak binding affinity for the HIC column, was eluted first, followed by the elution of the exendin-4-immunoglobulin Fc sample, which has a strong binding affinity, to the rear part. The separation was more easily performed compared with the ion exchange column due to the larger difference in hydrophobicity.

[0192]

Column: SOURCE Q (XK 16 mL, Amersham Biosciences)

Flow rate: 2.0 mL/min

Gradient: A0 ->25% 70 min B (A: 20 mM Tris, pH 7.5, B: A+ 1 M NaCl)

Column: SOURCE ISO (HR 16 mL, Amersham Biosciences)

Flow rate: 7.0 mL/min

Gradient: B 100 -> 0% 60 min B [A: 20 mM Tris (pH 7.5), B: A + 1.5 M ammonium sulfate ((NH₄)₂SO₄)]

[0193] The thus-prepared conjugate, in which the exendin-4 derivative and the immunoglobulin Fc region were linked by PEG, was named as "a long-acting exendin-4 derivative". Also, such term can be interchangeable used with "a long-acting exendin derivative".

Experimental Example 1: Effect of body weight reduction in rats with high fat diet-induced obesity

[0194] In this experiment, high-fat diet-induced obesity rats, which are widely used as obesity animal models, were used. The body weight of the rats before administration was about 600 g. The rats were housed individually during the experiment and were given *ad libitum* access to water. Lighting was not provided between 6 AM and 6 PM.

[0195] The test groups fed with high-fat diet include: Group 1, with an excipient (injection once every 3 days) - control group; Group 2, the long-acting exendin derivative of Example 6 at 3.3 nmol/kg (injection once every 3 days); Group 3, the long-acting derivative of SEQ ID NO: 12 at 1.6 nmol/kg (injection once every 3 days); Group 4, the long-acting derivative of SEQ ID NO: 12 at 3.3 nmol/kg (injection once every 3 days); Group 5, the long-acting derivative of SEQ ID NO: 12 at 6.6 nmol/kg (injection once every 3 days); Group 6, the long-acting exendin derivative of Example 6 at 3.3 nmol/kg + the long-acting derivative of SEQ ID NO: 12 at 1.6 nmol/kg (injection once every 3 days, respectively); Group 7, the long-acting exendin derivative of Example 6 at 3.3 nmol/kg + the long-acting derivative of SEQ ID NO: 12 at 3.3 nmol/kg (injection once every 3 days, respectively); Group 8, the long-acting exendin derivative of Example 6 at 3.3 nmol/kg + the long-acting derivative of SEQ ID NO: 12 at 6.6 nmol/kg (injection once every 3 days, respectively); Group 9, a paired-feeding with Group 4; and Group 10, a paired-feeding with Group 7. The experiment was terminated on the 15th day, and the changes in body weight of the rats in each group were measured at 3-day intervals during the progress of the experiment. Upon termination of the experiment, the amount of mesenteric fat

and liver weight were measured by autopsy. Statistical analysis was performed to compare between the excipient group (control group) and test groups by 1-way ANOVA.

[0196] As a result of the measurement of changes in body weight, as can be confirmed in FIG. 1, the groups administered with either the long-acting exendin derivative or the long-acting derivative of SEQ ID NO: 12 alone showed a decrease in body weight by - 8% and -7% to -22%, compared to that before administration, whereas in groups with a combined administration of the long-acting exendin derivative and the long-acting derivative of SEQ ID NO: 12, the effect of reducing body weight was improved further from -22% to -35%.

[0197] Additionally, when the effect of a body weight decrease in the group administered with the long-acting derivative of SEQ ID NO: 12 alone and the group administered with the combination of the long-acting exendin derivative and the long-acting derivative of SEQ ID NO: 12 was compared with that of the paired feeding group, respectively, a difference of about -11% and about -17% was shown, respectively, thus confirming that the body weight reducing effect was shown when administered with the glucagon derivative alone or the combined administration, by actions other than dietary intake.

[0198] That is, it was confirmed that the long-acting glucagon derivative of the present invention could play an additional role in body weight reduction in addition to the effect of anorexia.

[0199] Additionally, as a result of the measurement of the amount of mesenteric fat and liver weight, as can be confirmed in FIGS. 2 and 3, the combined administration of the long-acting exendin derivative and the long-acting derivative of SEQ ID NO: 12 showed a significant decrease in body fat and also a decrease in the weight of the liver compared to that of the group administered with an excipient. In particular, the increase/decrease of the weight of the liver is generally caused by the increase/decrease of the fat present in the liver, and the above effect of decrease in the weight of the liver shows the effect of reducing the liver fat. Accordingly, the decrease of the fat in the liver can be measured as a method for measuring the therapeutic effect of metabolic syndrome such as obesity, diabetes, nonalcoholic steatohepatitis, etc.

Experimental Example 2: Effect of body weight reduction in mice with high fat diet-induced obesity

[0200] In this experiment, high-fat diet-induced obesity mice, which are widely used as obesity animal models, were used. The body weight of the mice before administration was about 55 g. The mice were housed 7 mice per each group during the experiment and were given *ad libitum* access to water. Lighting was not provided between 6 AM and 6 PM.

[0201] The test groups fed with high-fat diet include: Group 1, with an excipient (injection once every 2 days) - control group; Group 2, the long-acting exendin derivative of Example 6 at 4.3

nmol/kg (injection once every 2 days); Group 3, the long-acting derivative of SEQ ID NO: 20 at 4.4 nmol/kg (injection once every 2 days); Group 4, the long-acting derivative of SEQ ID NO: 20 at 8.8 nmol/kg (injection once every 2 days); Group 5, the long-acting exendin derivative of Example 6 at 4.3 nmol/kg + the long-acting derivative of SEQ ID NO: 20 at 4.4 nmol/kg (injection once every 2 days); Group 6, the long-acting exendin derivative of Example 6 at 2.1 nmol/kg + the long-acting derivative of SEQ ID NO: 20 at 6.6 nmol/kg (injection once every 2 days); and Group 7, the long-acting exendin derivative of Example 6 at 0.8 nmol/kg + the long-acting derivative of SEQ ID NO: 20 at 8.0 nmol/kg (injection once every 2 days). The experiment was terminated on the 22nd day, and the changes in body weight of the mice in each group were measured at 2-day intervals during the progress of the experiment. Upon termination of the experiment, the weight of the mouse livers was measured by autopsy.

[0202] As a result of the measurement of changes in body weight, as can be confirmed in FIG. 4, each of the groups administered with the long-acting derivative of SEQ ID NO: 20 (8.8 nmol/kg, injection once every 2 days) alone showed a decrease in body weight by -25% and -29%, respectively, compared to that before administration. Additionally, the effect of reducing body weight was shown to increase further when administered in combination with the long-acting exendin derivative. It was also confirmed that the combined administration of the long-acting exendin derivative and the long-acting derivative of SEQ ID NO: 20 at a ratio of 1:1, 1:3, and 1:10 further increased the effect of reducing body weight by -50% or higher. Additionally, the effect of reducing body weight according to the ratio between the long-acting exendin derivative and the long-acting derivative of SEQ ID NO: 20 was not significant, however, the effect of anorexia became higher along with the increase in the percentage of the long-acting exendin derivative, thus confirming that the glucagon long-acting derivative could play an additional role in body weight reduction in addition to the effect of anorexia.

[0203] Additionally, as a result of the measurement of the total cholesterol in the blood, as can be confirmed in FIG. 5, each of the groups administered with the long-acting exendin derivative (4.4 nmol/kg, injection once every 2 days) and the long-acting derivative of SEQ ID NO: 20 (8.8 nmol/kg, injection once every 2 days) showed a decrease in cholesterol by -35% and -71%, respectively. From the above, it was confirmed that the glucagon long-acting derivative could play an additional role in reducing blood cholesterol in addition to the effect of anorexia. Statistical analysis was performed to compare between the excipient group (control group) and test groups by 1-way ANOVA.

[0204] The scope of the present invention is indicated by the appended claims rather than by the foregoing description.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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PATENTKRAV

1. Farmaceutisk sammensætning til anvendelse i en fremgangsmåde til behandling eller forebyggelse af metaboliske syndromer omfattende i) et peptid omfattende aminosyresekvensen ifølge SEQ ID NO: 20 eller 37 og ii) et insulinotropt peptid, hvor peptidet omfattende aminosyresekvensen ifølge SEQ ID NO: 20 eller 37 er i form af et langtidsvirkende konjugat, hvortil et biokompatibelt materiale, der er i stand til at øge peptidets *in vivo*-halveringstid, er bundet, og det insulinotrope peptid er i form af et langtidsvirkende konjugat, hvortil et biokompatibelt materiale, der er i stand til at øge det insulinotrope peptids *in vivo*-halveringstid, er bundet.
2. Farmaceutisk sammensætning til anvendelse ifølge krav 1, hvor peptidet, der omfatter aminosyresekvensen ifølge SEQ ID NO: 20 eller 37, har en isoelektrisk punktværdi (pI), der er forskellig fra den for nativ glukagon (6.8) eller er et glukagonderivat, der er i stand til at aktivere en glukagonreceptor.
3. Farmaceutisk sammensætning til anvendelse ifølge krav 1, hvor det insulinotrope peptid er valgt fra gruppen bestående af GLP-1, exendin-3, exendin-4, en agonist deraf, et derivat deraf, et fragment deraf, en variant deraf og en kombination deraf; og eventuelt hvor det insulinotrope peptid er et insulinotropt peptidderivat, hvor den N-terminale histidinrest er substitueret med en valgt fra gruppen bestående af desamino-histidyl, N-dimethyl-histidyl, β -hydroxyimidazopropionyl, 4-imidazoacetyl og β -carboxyimidazopropionyl.
4. Farmaceutisk sammensætning til anvendelse ifølge krav 3, hvor det insulinotrope peptid er valgt fra gruppen bestående af en nativ exendin-4; et exendin-4-derivat, hvor den N-terminale amingruppe af exendin-4 er deleteret; et exendin-4-derivat, hvor den N-terminale amingruppe af exendin-4 er substitueret med en hydroxylgruppe; et exendin-4-derivat, hvor den N-terminale amingruppe af exendin-4 er modificeret med en dimethylgruppe; et exendin-4-derivat, hvor α -carbonet i den første aminosyre af exendin-4, histidin, er deleteret; et exendin-4-derivat, hvor den 12. aminosyre af exendin-4, lysin, er substitueret med serin; og et exendin-4-derivat, hvor den 12. aminosyre af exendin-4, lysin, er substitueret med arginin.
5. Farmaceutisk sammensætning til anvendelse ifølge krav 1, hvor

(i) det biokompatible materiale er valgt fra gruppen bestående af polyethylenglycol, fedtsyre, kolesterol, albumin og et fragment deraf, et albuminbindende materiale, en polymer af gentagne enheder af en bestemt aminosyresekvens, et antistof, et antistoffragment, et FcRn-bindende materiale, *in vivo* bindevæv eller et derivat deraf, et nukleotid, fibronectin, transferrin, et saccharid og en polymer; eller

(ii) peptidet omfattende aminosyresekvensen ifølge SEQ ID NO: 20 eller 37 og det insulinotrope peptid er henholdsvis bundet til et biokompatibelt materiale ved hjælp af en linker valgt fra gruppen bestående af polyethylenglycol, polypropylenglycol, en ethylenglycol-propylenglycol-copolymer, polyoxyethyleret polyol, polyvinylalkohol, et polysaccharid, dextran, polyvinylethylether, en bionedbrydelig polymer, herunder polymælkesyre (PLA) eller polymælke-glykolsyre (PLGA), lipidpolymer, kitin, hyaluronsyre, fedtsyre, en polymer, en lavmolekylær forbindelse, et nukleotid og en kombination deraf.

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6. Farmaceutisk sammensætning til anvendelse ifølge krav 1,

hvor det biokompatible materiale er et FcRn-bindende materiale, og peptidet omfattende aminosyresekvensen SEQ ID NO: 20 eller 37 og det insulinotrope peptid er henholdsvis bundet til et biokompatibelt materiale ved hjælp af en peptidlinker eller en ikke-peptidlinker valgt fra gruppen bestående af polyethylenglycol, polypropylenglycol, en ethylenglycol-propylenglycol-copolymer, polyoxyethyleret polyol, polyvinylalkohol, et polysaccharid, dextran, polyvinylethylether, en bionedbrydelig polymer, herunder polymælkesyre (PLA) eller polymælke-glykolsyre (PLGA), lipidpolymer, kitin, hyaluronsyre og en kombination deraf; og eventuelt hvor det FcRn-bindende materiale er et polypeptid, der omfatter en immunglobulin-Fc-region.

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7. Farmaceutisk sammensætning til anvendelse ifølge krav 6, hvor

- (i) immunoglobulin-Fc-regionen er aglycosyleret;
- (ii) immunoglobulin-Fc-regionen er valgt fra gruppen bestående af:
 - (a) et CH1-domæne, et CH2-domæne, et CH3-domæne og et CH4-domæne;
 - (b) et CH1-domæne og et CH2-domæne;
 - (c) et CH1-domæne og et CH3-domæne;
 - (d) et CH2-domæne og et CH3-domæne;

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- (e) en kombination mellem ét eller mindst to domæner blandt et CH1-domæne, et CH2-domæne, et CH3-domæne og et CH4-domæne og en immunglobulin-hængselregion eller en del af hængselregionen; og
- (f) en dimer mellem hvert domæne af den tunge kædes konstante region og den lette kædes konstante region;
- (iii) polypeptidet, der omfatter immunoglobulin-Fc-regionen, er i form af en dimer;
- (iv) immunglobulin-Fc-regionen er et nativt Fc-derivat, hvor regionen, der er i stand til at danne en disulfidbinding, er deleteret, et nativt Fc-derivat, hvor en del af aminosyren/aminosyrerne i N-terminalen er fjernet, et nativt Fc-derivat, hvor et methionin er tilføjet til N-terminalen, et nativt Fc-derivat, hvor et komplekmentbindingssted er deleteret, eller et nativt Fc-derivat, hvor et antistofafhængigt cellededieret cytotoxicitets-(ADCC)-sted er deleteret;
- (v) immunglobulin-Fc-regionen er afledt af et immunoglobulin valgt fra gruppen bestående af IgG, IgA, IgD, IgE og IgM, og eventuelt hvor immunoglobulin-Fc-regionen er en IgG4-Fc-region; eller
- (vi) immunoglobulin-Fc-regionen er en aglycosyleret Fc-region afledt af humant IgG4.
8. Farmaceutisk sammensætning til anvendelse ifølge krav 6, hvor
- (i) ikke-peptidlinkeren er bundet til cysteinresten af peptidet omfattende aminosyresekvensen ifølge SEQ ID NO: 20 eller 37; eller
- (ii) begge ender af ikke-peptidlinkeren er henholdsvis bundet til en aminogruppe eller en thiolgruppe af peptidet, som omfatter aminosyresekvensen ifølge SEQ ID NO: 20 eller 37, eller det insulinotrope peptid og det biokompatible materiale.
9. Farmaceutisk sammensætning til anvendelse ifølge krav 1, hvor det metaboliske syndrom er valgt fra gruppen bestående af nedsat glukosetolerance, hyperkolesterolæmi, dyslipidæmi, fedme, diabetes, hypertension, ikke-alkoholisk steatohepatitis (NASH), åreforkalkning forårsaget af dyslipidæmi, aterosklerose, arteriosklerose, koronar hjertesygdom og slagtilfælde.
10. Isoleret peptid omfattende aminosyresekvensen ifølge SEQ ID NO: 37.

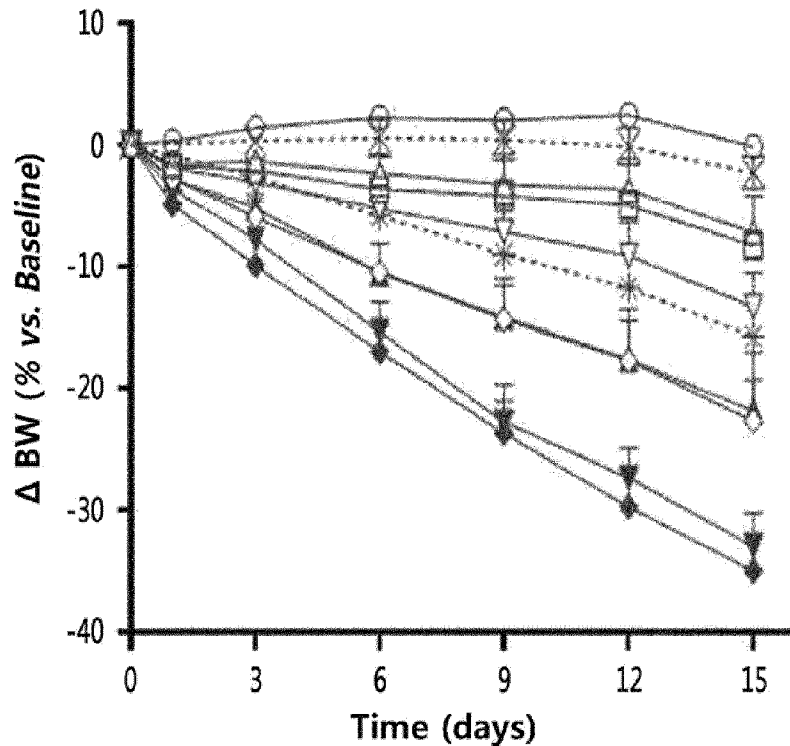
11. Isoleret polynukleotid, der koder for det isolerede peptid ifølge krav 10.
12. Vektor omfattende det isolerede polynukleotid ifølge krav 11.
- 5 13. Isoleret konjugat, hvor det isolerede peptid ifølge krav 10 og et biokompatibelt materiale, der er i stand til at øge *in vivo*-halveringstid, er forbundet.
14. Isoleret konjugat ifølge krav 13, hvor
- 10 (i) det biokompatible materiale er valgt fra gruppen bestående af polyethylenglycol, fedtsyre, kolesterol, albumin og et fragment deraf, et albuminbindende materiale, en polymer af gentagne enheder af en bestemt aminosyresekvens, et antistof, et antistoffragment, et FcRn-bindende materiale, *in vivo* bindevæv eller et derivat deraf, et nukleotid, fibronectin, transferrin, et saccharid og en polymer; eller
- 15 (ii) peptidet er bundet til et biokompatibelt materiale af en linker valgt fra gruppen bestående af polyethylenglycol, polypropylenglycol, en ethylenglycol-propylenglycol-copolymer, polyoxyethyleret polyol, polyvinylalkohol, et polysaccharid, dextran, polyvinylethylether, en bionedbrydelig polymer, herunder polymælkesyre (PLA) eller polymælke-glycolsyre (PLGA), lipidpolymer, chitin, hyaluronsyre,
- 20 syre, fedtsyre, en polymer, en lavmolekylær forbindelse, et nukleotid og en kombination deraf.
15. Isoleret konjugat ifølge krav 13,
- 25 hvor det biokompatible materiale er et FcRn-bindende materiale, og det isolerede peptid er bundet til et biokompatibelt materiale af en peptidlinker eller en ikke-peptidlinker valgt fra gruppen bestående af polyethylenglycol, polypropylenglycol, en ethylenglycol-propylenglycol-copolymer, polyoxyethyleret polyol, polyvinylalkohol, et polysaccharid, dextran, polyvinylethylether, en bionedbrydelig polymer, herunder polymælkesyre (PLA) eller polymælke-glycolsyre (PLGA), lipidpolymer, chitin, hyaluronsyre og en kombination deraf; eventuelt hvor det FcRn-bindende materiale er et polypeptid, der omfatter en immunoglobulin-Fc-region.
- 30

16. Sammensætning omfattende det isolerede peptid ifølge krav 10 eller det isolerede konjugat ifølge krav 15.

5 17. Farmaceutisk sammensætning til anvendelse i en fremgangsmåde til behandling eller forebyggelse af hypoglykæmi eller metabolisk syndrom, hvor sammensætningen omfatter det isolerede peptid ifølge krav 10 eller det isolerede konjugat ifølge krav 13.

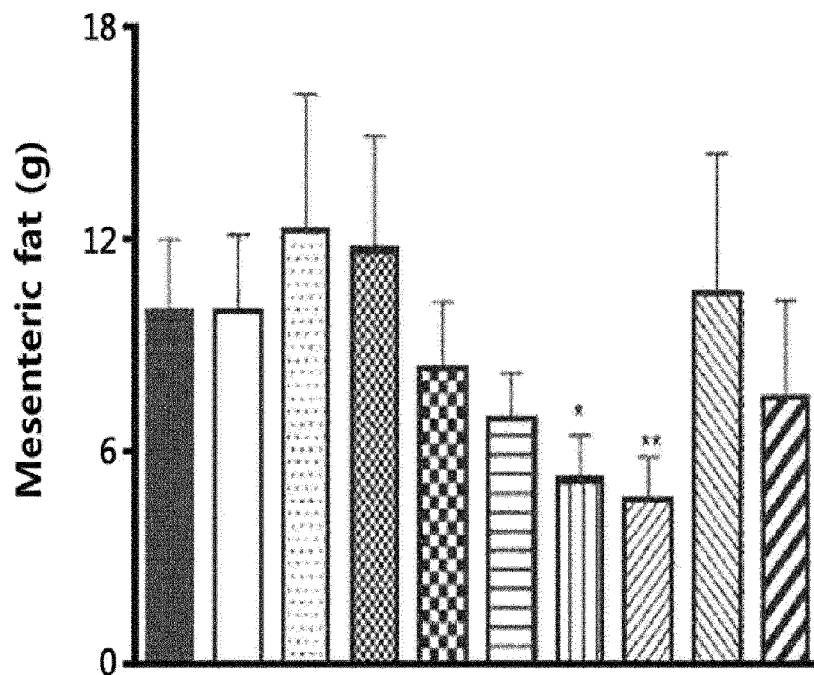
DRAWINGS

【Figure 1】



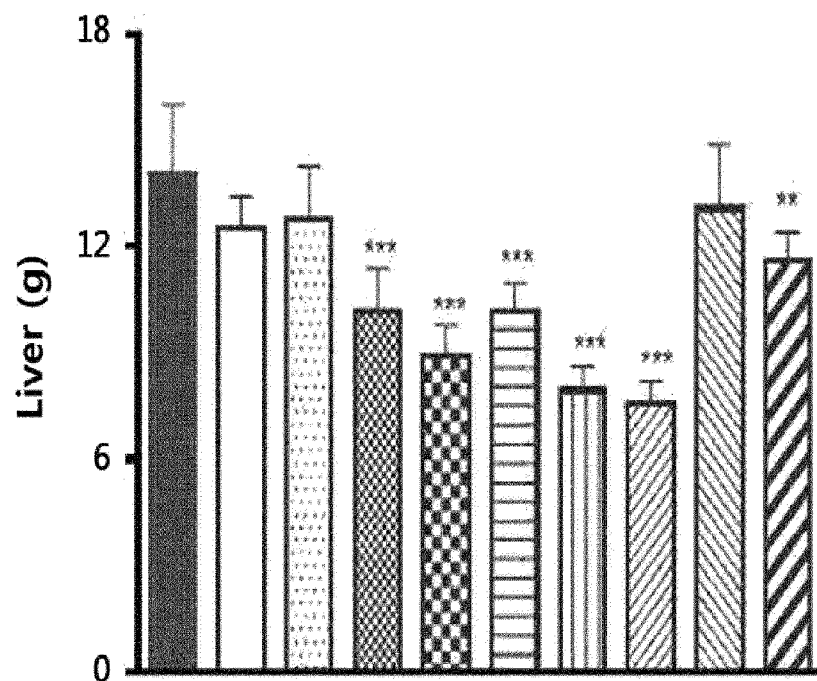
- excipient
- long-acting exendin-4 derivative (3.3 nmol/kg)
- △ long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▽ long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ◇ long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- ★ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▼ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ◆ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- × paired feeding (to long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))
- ※ paired feeding (to long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))

【Figure 2】



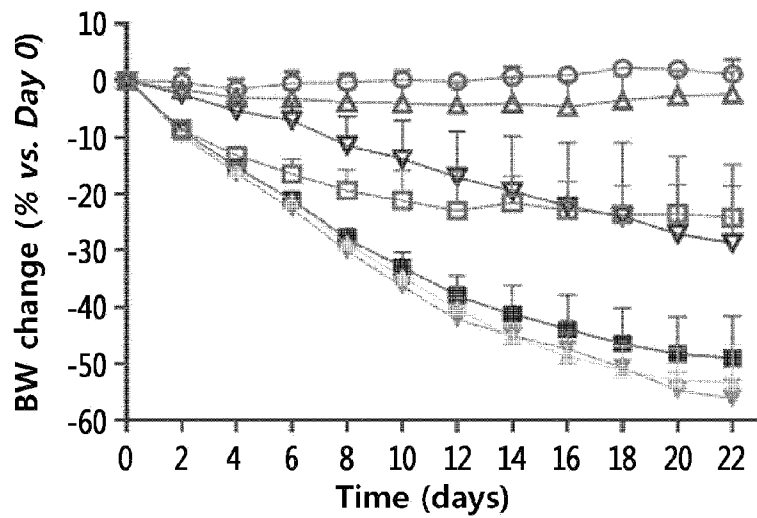
- excipient
- long-acting exendin-4 derivative (3.3 nmol/kg)
- ▨ long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▩ long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ▧ long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- ▦ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▤ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ▣ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- ▢ paired feeding (to long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))
- paired feeding (to long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))

【Figure 3】



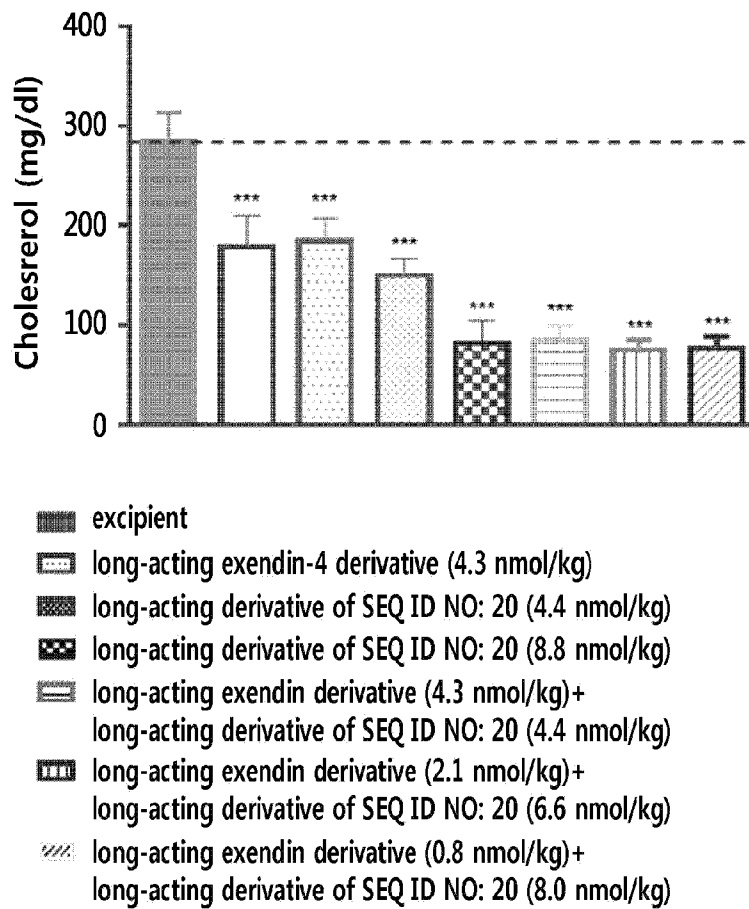
- excipient
- long-acting exendin-4 derivative (3.3 nmol/kg)
- ▨ long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▩ long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ▧ long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- ▦ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (1.6 nmol/kg)
- ▤ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg)
- ▣ long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (6.6 nmol/kg)
- ▢ paired feeding (to long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))
- paired feeding (to long-acting exendin-4 derivative (3.3 nmol/kg)+
long-acting derivative of SEQ ID NO: 12 (3.3 nmol/kg))

[Fig. 4]



- excipient
- long-acting exendin-4 derivative (4.3 nmol/kg)
- △ long-acting derivative of SEQ ID NO: 20 (4.4 nmol/kg)
- ▽ long-acting derivative of SEQ ID NO: 20 (8.8 nmol/kg)
- ⊕ long-acting exendin derivative (4.3 nmol/kg) + long-acting derivative of SEQ ID NO: 20 (4.4 nmol/kg)
- ⊖ long-acting exendin derivative (2.1 nmol/kg) + long-acting derivative of SEQ ID NO: 20 (6.6 nmol/kg)
- ⊗ long-acting exendin derivative (0.8 nmol/kg) + long-acting derivative of SEQ ID NO: 20 (8.0 nmol/kg)

[Fig. 5]



SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

