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# Analyses of Insulin-Potentiating Fragments of Human Growth Hormone by Computative Simulation; Essential Unit for Insulin-Involved Biological Responses

Kazuto Ohkura[a,b,*] and Hitoshi Hori[a]

[a]*Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan*

[b]*Department of Applied Biological Sciences, Faculty of Agricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Aichi 464-8601, Japan*

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**Abstract**—We analyzed the structural features of insulin-potentiating fragments of human growth hormone by computative simulations. The peptides were designated from the N-terminus sequences of the hormone positions at 1–15 ($hGH_{1–15}$; $H_2N$-Phe[1]-Pro[2]-Thr[3]-Ile[4]-Pro[5]-Leu[6]-Ser[7]-Arg[8]-Leu[9]-Phe[10]-Asp[11]-Asn[12]-Ala[13]-Met[14]-Leu[15]-COOH), 6–13 ($hGH_{6–13}$), 7–13 ($hGH_{7–13}$) and 8–13 ($hGH_{8–13}$), which enhanced insulin-producing hypoglycemia. In these peptide molecules, ionic bonds were predicted to form between 8th-arginyl residue and 11th-aspartic residue, and this intramolecular interaction caused the formation of a macrocyclic structure containing a tetrapeptide Arg[8]-Leu[9]-Phe[10]-Asp[11]. The peptide positions at 6–10 ($hGH_{6–10}$), 9–13 ($hGH_{9–13}$) and 10–13 ($hGH_{10–13}$) did not lead to a macrocyclic formation in the molecules, and had no effect on the insulin action. Although β-Ala[13]$hGH_{1–15}$, in which the 13th-alanine was replaced by a β-alanyl residue, had no effect on insulin-producing hypoglycemia, the macrocyclic region (Arg[8]-Leu[9]-Phe[10]-Asp[11]) was observed by the computative simulation. An isothermal vibration analysis of both of β-Ala[13]$hGH_{1–15}$ and $hGH_{1–15}$ peptide suggested that β-Ala[13]$hGH_{1–15}$ molecule was more flexible than $hGH_{1–15}$; C-terminal carboxyl group of Leu[15] easily accessed to Arg[8] and inhibited the ionic bond formation between Arg[8] and Asp[11] in β-Ala[13]$hGH_{1–15}$. The peptide of $hGH_{8–13}$ dose-dependently enhanced the insulin-involved fatty acid synthesis in rat white adipocytes, and stabilized the $C_6$-NBD-PC (1-acyl-2-[6-[(7-nitro-2,1,3benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphatidylcholine) model membranes. In contrast, $hGH_{9–13}$ had no effect both on the fatty acid synthesis and the membrane stability. In the same culture conditions as the fatty acid synthesis assay, $hGH_{8–13}$ had no effect on the transcript levels of glucose transporter isoforms (GLUT 1, 4) and hexokinase isozymes (HK I, II) in rat white adipocytes. Judging from these results we considered that the macrocyclic structure in human growth hormonal peptides is regarded with the modification of insulin action, and $hGH_{8–13}$ is an essential sequence for the modification of insulin action. This $hGH_{8–13}$ peptide modifies the insulin action via stabilizing the cell membrane, and does not directly act on the insulin-involved glucose metabolism. 

## Introduction

Insulin resistance is a term used when a given concentration of insulin does not elicit a normal biological response. Non-insulin-dependent diabetes mellitus (NIDDM) is the disease classically associated with the insulin resistance, but the insulin resistance also occurs in other common conditions including obesity, aging, polycystic ovarian syndrome, hypertension, hyperinsulinemia, dyslipidemia and cardiovascular disease.[1–3] Thus, insulin resistance is a complex and multifactorial result of a variety of genetic, cellular and environmental causes.[1–3] In the present study, we analyzed the structural features of hGH peptides, which modify the insulin action described in the following, to reveal the essential structure for modification of insulin action.

The functional domain of human growth hormone (hGH) for the insulin-potentiating action was first demonstrated to be located at the amino-terminus of the molecule by Ng et al.[4] This has subsequently been confirmed by the isolation of a 5-kDa peptide, the amino-terminal fragment $hGH_{1–43}$, from human pituitary gland.[5] A series of hGH peptide fragments including $hGH_{1–43}$, $hGH_{1–20}$, $hGH_{1–15}$, $hGH_{4–15}$ and $hGH_{6–13}$ was tested and found to induce hypoglycemia in vivo and to potentiate the actions of insulin in vitro.[4,6,7] The amino-terminal peptides of $hGH_{1–15}$, $hGH_{6–13}$, $hGH_{7–13}$ and $hGH_{8–13}$ had

*Corresponding author at first address: Fax: +81-88-656-7525; e-mail: kohkura@sag.bekkoame.ne.jp

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been known as the insulin-potentiating fragments, and increased the binding of insulin to its specific receptor. Thus, the peptides modulated the action of glycogen synthase and phosphorylase, and produced hypoglycemia as a result of the increase of glycogen storage in adipose tissue.[8] On the contrary, the amino-terminal fragments including $hGH_{6–10}$, $hHG_{9–13}$ and $hGH_{10–13}$ had no effect on these insulin actions, e.g., insulin-producing hypoglycemia.[8] Moreover, the fragment of β-$Ala^{13}$ h $GH_{1–15}$, in which the 13th-alanine was replaced by a β-alanyl residue, had no effect on the insulin action.[8] The effects of these hGH peptides on insulin-producing hypoglycemia and the insulin binding to the hepatic plasma membranes are summarized in Table 1.

It is easily considered that these hGH fragment peptides have several conformations in aqueous solution. In the present study, we performed the molecular mechanics (MM) computation and the molecular dynamics (MD) simulations for these insulin-potentiating hGH peptides ($hGH_{1–15}$, $hGH_{6–13}$, $hGH_{7–13}$, $hGH_{8–13}$) and other peptides ($hGH_{6–10}$, $hGH_{9–13}$, $hGH_{10–13}$, β-$Ala^{13}hGH_{1–15}$), and we analyzed the three-dimensional structural features of the insulin-potentiating peptides. Moreover, we estimated the essential unit of hGH peptide for the potentiation of insulin action, and we examined the effect of the unit on the insulin-involved fatty acid synthesis in rat white adipocytes.

In general, cellular metabolic changes induced by hormones have been evidenced to accompany the modifications of cell membranes.[9–13] In the insulin resistance, the alterations of molecular organizations in the cell membranes are expected to closely relate to their metabolic regulations of the cells. Then we examined the effect of hGH peptide on the membrane stability by using 1-acyl-2-[6-[(7-nitro-2,1,3benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphatidylcholine ($C_6$-NBD-PC) model membranes, and discussed the relationships between the membrane condition and the cell responses to insulin (e.g., the insulin-involved fatty acid synthesis).

**Table 1.** Effect of synthetic hGH peptides in rats during intravenous insulin tolerance tests and insulin binding to hepatic plasma membrane (summarized from ref 8)[a]

| | Reduced blood glucose (mM) | | |
|---|---|---|---|
| | 30 min | 60 min | Bound insulin (%) |
| Control | 1.1 | 0.9 | 60 |
| $hGH_{1–15}$ | 2.0 | 1.9 | 95 |
| $hGH_{6–13}$ | 1.6 | 1.5 | 85 |
| $hGH_{7–13}$ | 1.7 | 1.6 | 85 |
| $hGH_{8–13}$ | 1.6 | 1.5 | 85 |
| $hGH_{6–10}$ | 1.1 | 0.8 | 60 |
| $hGH_{9–13}$ | 1.1 | 0.8 | 60 |
| $hGH_{10–13}$ | 1.1 | 0.9 | 60 |
| β-$Ala^{13}hGH_{1–15}$ | 1.1 | 1.0 | 60 |

[a]Peptides (40 μM) were administered intravenously 5 min before the insulin (0.10 U/kg body weight) injection. Blood samples for glucose analysis were taken 30 and 60 min after the insulin injection, and the net glucose concentration reduced in blood was determined. Isolated hepatic plasma membranes were preincubated with or without synthetic hGH peptides (40 μM) in 50 mM Tris–HCl buffer (pH 7.5) containing 0.5% bovine serum albumin for 15 min at 25 °C, and resuspended in the buffer containing [$^{125}$I] insulin for a further 30 min at 25 °C. The percentage of insulin bound was determined.

A glucose metabolism, the uptake of glucose and its phosphorylation are major cellular responses to insulin. In mammalian cells, four isoforms of glucose transporter (GLUT 1–GLUT 4) are known to facilitate the uptake of glucose through the plasma membranes.[14–18] In addition, the first steps of the sequential reactions in glucose metabolism are catalyzed by four isozymes of hexokinase (HK I–HK IV).[19,20] Thus GLUT and HK are important factors in the insulin-involved glucose metabolism. Therefore, we analyzed the effect of hGH peptide on the transcript levels of GLUT isoforms and HK isozymes in white adipocytes.

## Results

### Computative simulations of three-dimensional structure of hGH peptides

The effects of amino-terminal fragments of human growth hormone (hGH) on both insulin-producing hypoglycemia and the insulin binding to the hepatic plasma membranes are summarized in Table 1.[8] Based on the crystallographic geometry of growth hormone and its receptor complex, the amino-terminal region of heavy chain of the hormone contacts the receptor protein.[21] Figure 1A shows the three-dimensional structure of amino-terminus of growth hormone ($hGH_{1–15}$; $H_2N$-$Phe^1$-$Pro^2$-$Thr^3$-$Ile^4$-$Pro^5$-$Leu^6$-$Ser^7$-$Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$-$Asn^{12}$-$Ala^{13}$-$Met^{14}$-$Leu^{15}$-COOH), which was determined by the global minimum search using CONFLEX. The 8th-arginyl residue formed an ionic bond to the 11th-aspartic residue, and this intramolecular interaction made a macrocyclic structure comprised of the tetrapeptide, $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ (bold line). A macrocyclic structure of $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ region (bold line) was always found in the fragments of $hGH_{6–13}$ (Fig. 1B), $hGH_{7–13}$ (Fig. 1C) and $hGH_{8–13}$ (Fig. 1D) by the global minimum search in the same way as with $hGH_{1–15}$, and these fragments produced the insulin action (Table 1). In the hexapeptide ($hGH_{8–13}$), the length between the guanidyl carbon of 8th-arginine ($Arg^8$ in Fig. 1D) and the carboxylic carbon of 11th-aspartic acid ($Asp^{11}$ in Fig. 1D) was determined as 3.1 angstrom.

The peptides of $hGH_{6–10}$ (Fig. 2A), $hGH_{9–13}$ (Fig. 2B) and $hGH_{10–13}$ (Fig. 2C) never form a macrocyclic structure: $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$, and these peptides had no effect on insulin-producing hypoglycemia (Table 1). Although a macrocyclic structure was observed in β-$Ala^{13}hGH_{1–15}$ (Fig. 2D, bold line), in which the 13th-alanine was replaced by a β-alanyl residue, it had no effect on the insulin action (Table 1). Comparing the conformation of β-$Ala^{13}hGH_{1–15}$ and $hGH_{1–15}$ peptide, $Arg^8$ of β-$Ala^{13}hGH_{1–15}$ was shown to bind not only to the carboxyl group of $Asp^{11}$ but also to the carboxyl group of $Leu^{15}$ (Fig. 2D versus 1A). Figure 3 shows the energy profiles of β-$Ala^{13}hGH_{1–15}$ and $hGH_{1–15}$ through their holding processes. In isothermal vibration analysis, the energy level of β-$Ala^{13}hGH_{1–15}$ arrived at

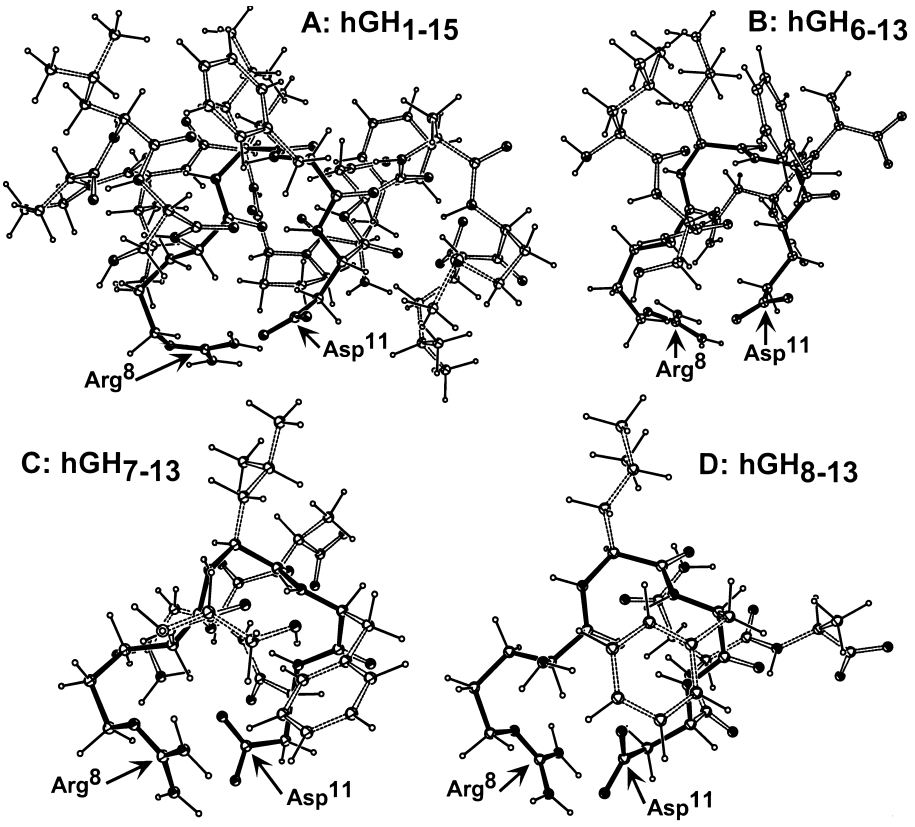


**Figure 1.** Three-dimensional structure of amino-terminal peptides of human growth hormone. The optimized 3-D-projected view of $hGH_{1–15}$: $H_2N$-Phe[1]-Pro[2]-Thr[3]-Ile[4]-Pro[5]-Leu[6]-Ser[7]-Arg[8]-Leu[9]-Phe[10]-Asp[11]-Asn[12]-Ala[13]-Met[14]-Leu[15]-COOH (A), $hGH_{6–13}$ (B), $hGH_{7–13}$ (C) and $hGH_{8–13}$ peptide (D). The macrocyclic ring ($Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$) is indicated as a bold line. The guanidyl carbon of 8th-arginine ($Arg^8$) and the carboxylic carbon of 11th-aspartic acid ($Asp^{11}$) are indicated with arrows.

an equivalent state for 5 ps (Fig. 3A), while the energy of $hGH_{1–15}$ gradually decreased to an equivalent state (Fig. 3B). This result suggests that the β-$Ala^{13}hGH_{1–15}$ molecule was more flexible than $hGH_{1–15}$. Then C-terminal carboxyl group of $Leu^{15}$ easily accessed to $Arg^8$ and inhibited the ionic bond formation between $Arg^8$ and $Asp^{11}$ in β-$Ala^{13}hGH_{1–15}$.

### Regulation of the insulin-involved fatty acid synthesis by $hGH_{8–13}$ peptide including a macrocyclic structure

Various β-agonistic drugs (such as epinephrine and isoproterenol) stimulate the triglyceride lipolysis in white adipose cells which have been designed and synthesized.[22–27] Insulin effectively counteracts this β-agonist-stimulated activity.[28] In rat white adipocytes, the stimulatory effect of isoproterenol on the lipolysis was antagonized by insulin (open circles in Fig. 4A). The amino-terminal hexapeptide ($hGH_{8–13}$; $H_2N$-$Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$-$Asn^{12}$-$Ala^{13}$-COOH) of human growth hormone (30 μM) enhanced the insulin action, and suppressed the fatty acid synthesis (closed circles in Fig. 4A). The hexapeptide increased the affinity of adipocytes for insulin up to 24.4-fold ($K_m$ values in Table 2). In the presence of insulin ($1.22\times10^{-11}$ M), the hexapeptide suppressed the fatty acid synthesis dose-dependently (closed circles, Fig. 4B), while it did not affect the fatty acid synthesis without insulin (open circles). The pentapeptide ($hGH_{9–13}$; $H_2N$-$Leu^9$-$Phe^{10}$-$Asp^{11}$-$Asn^{12}$-$Ala^{13}$-COOH) (30 μM) had no effect on the insulin-involved fatty acid synthesis (open triangles in Fig. 4A). The $K_m$ value was similar to the control value, and the ratio was 0.91 (Table 2). Moreover, dose-dependence of $hGH_{9–13}$ pentapeptide on the insulin-involved fatty acid synthesis was not observed without (open circles) or with (closed circles) insulin ($1.22\times10^{-11}$ M) (Fig. 4C).

### Effect of $hGH_{8–13}$ and $hGH_{9–13}$ peptides on the stability of a model membrane

When $C_6$-NBD-PC (1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphatidylcholine) micelles were incubated at 37 °C in 0.1 M Tris–HCl buffer (pH 7.4), NBD molecules were spontaneously released by the hydrolysis of phosphatidylcholine and the fluorescent intensity increased by the rate of 0.515 arbitrary unit per min (Fig. 4D; control). In the presence of 10 μM $hGH_{8–13}$, the $C_6$-NBD-PC model membrane was stabilized and the rate of hydrolysis (fluorescent increment) fell to 0.272 arbitrary unit per min (closed column). Indeed, $hGH_{8–13}$ dose-dependently suppressed the NBD liberation from $C_6$-NBD-PC micelles induced by phospholipase $A_2$ ($2.97\times10^{-6}$ M, at pH 8.3, in the presence of 20 mM $CaCl_2$) (data not shown). Ten micromoles of $hGH_{9–13}$ did not stabilize the $C_6$-NBD-PC model membrane, and the hydrolysis rate was almost unchanged compared to the control value (0.463 arbitrary unit per min; hatched column).

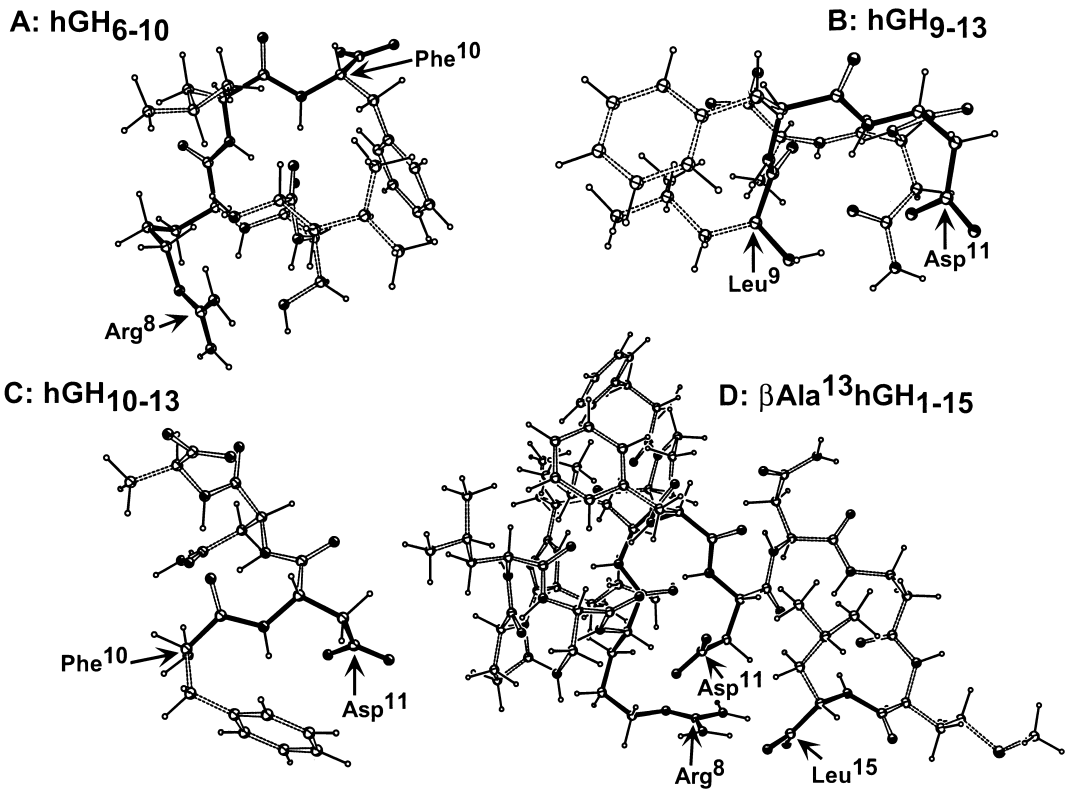


**Figure 2.** Three-dimensional structure of hGH peptides. The optimized 3-D-projected views of $hGH_{6–10}$ (A), $hGH_{9–13}$ (B) and $hGH_{10–13}$ (C). The regions at $Arg^8$-$Leu^9$-$Phe^{10}$, $Leu^9$-$Phe^{10}$-$Asp^{11}$ and $Phe^{10}$-$Asp^{11}$ in $hGH_{6–10}$, $hGH_{9–13}$ and $hGH_{10–13}$ are indicated as bold lines, respectively. β-$Ala^{13}hGH_{1–15}$ (D) had the 13th-alanine replaced by a β-alanyl residue. The macrocyclic region ($Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$) and the 15th-leucine are indicated as bold lines. The guanidyl carbon of 8th-arginine ($Arg^8$), the carboxylic carbon of 11th-aspartic acid ($Asp^{11}$) and the carboxylic carbon of 15th-leucine ($Leu^{15}$) are indicated with arrows.

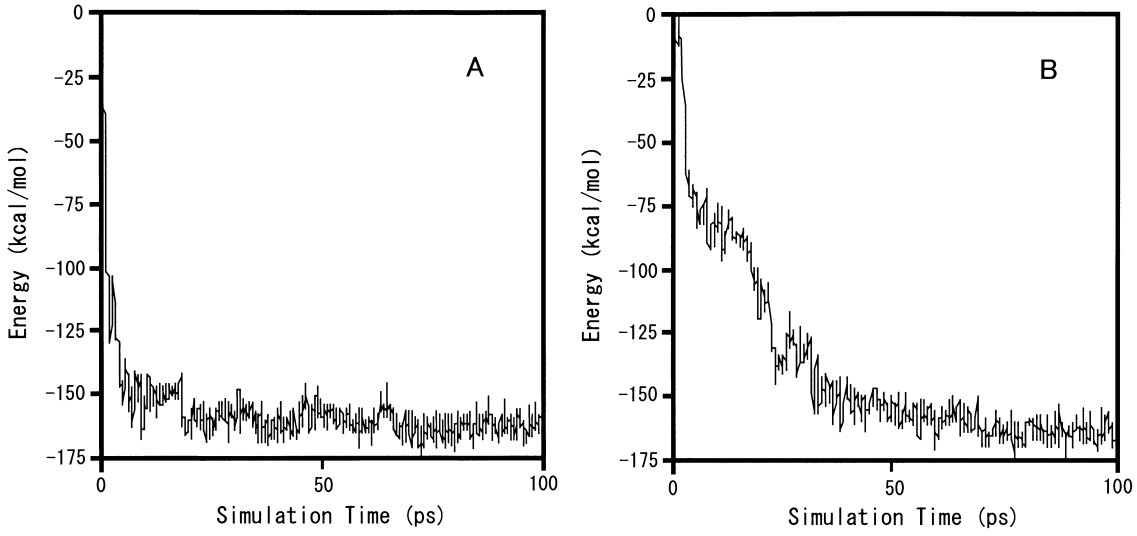


**Figure 3.** Energy profiles of β-$Ala^{13}hGH_{1–15}$ and $hGH_{1–15}$ through their holding processes. Isothermal vibrations of the β-$Ala^{13}$ including peptide (A; β-$Ala^{13}hGH_{1–15}$) and the intact peptide (B; $hGH_{1–15}$) were simulated with the geometry of the molecules protonated at pH 7.4 under the temperature of 300 K. Details are described in the Experimental section.

## Transcript levels of HK isozymes in white adipocytes

Next, we analyzed the transcript levels of four HK (I–IV) isozymes of rat white adipocytes in various culture conditions, which were the same as the conditions in the assay of insulin-involved fatty acid synthesis. Ten micrograms of RNA obtained from adipocytes were incubated as follows: without (condition 1) or with $1.22\times10^{-11}$ M insulin (condition 2), 2 nM isoproterenol (condition 3), $1.22\times10^{-11}$ M insulin and 2 nM isoproterenol (condition 4), $1.22\times10^{-11}$ M insulin and 2 nM isoproterenol and 20 μM $hGH_{8–13}$ (condition 5), 20 μM $hGH_{8–13}$ (condition

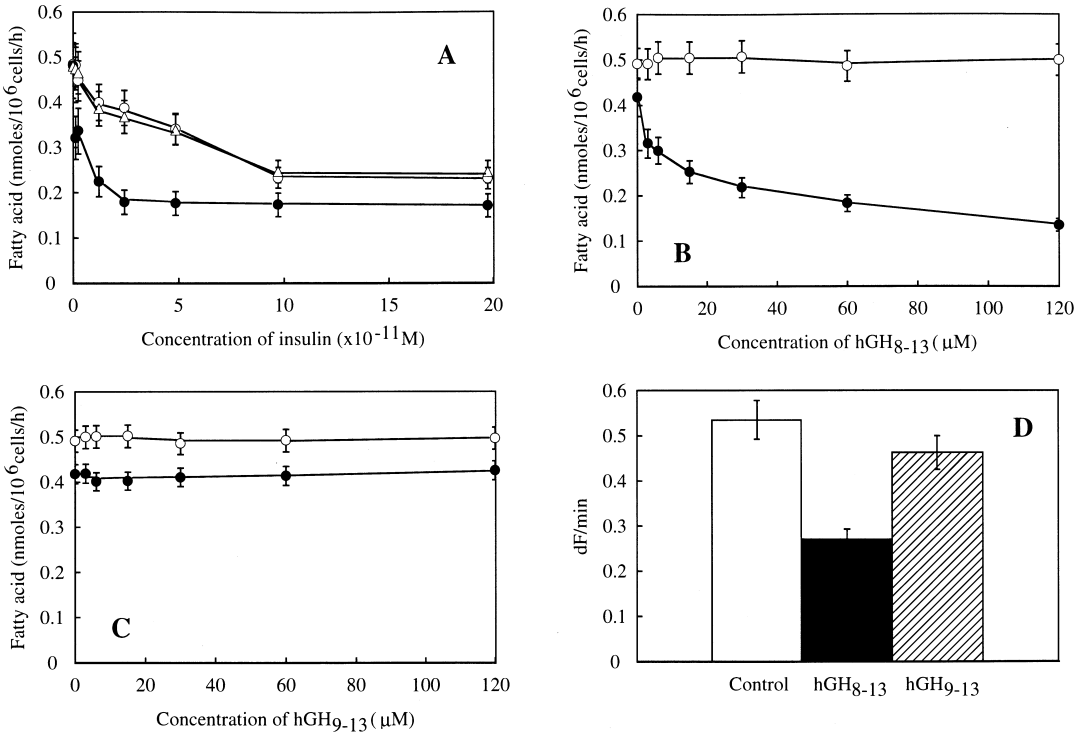


**Figure 4.** Effect of hGH peptides on insulin-involved fatty acid synthesis in rat white adipocytes and stability of model membrane. (A) Adipocytes were incubated at 37 °C for 2 h with 30 μM $hGH_{8–13}$ (closed circles), 30 μM $hGH_{9–13}$ (open triangles) or without (open circles) in the presence of 2 nM isoproterenol and various concentrations of insulin. (B), (C) Adipocytes were incubated at 37 °C for 2 h with $1.22\times10^{-11}$ M insulin (closed circles) or without insulin (open circles) in the presence of 2 nM isoproterenol and various amounts of $hGH_{8–13}$ (B) or $hGH_{9–13}$ (C). (D) $C_6$-NBD-PC was suspended at $1.29\times10^{5}$ M in 0.1 M Tris–HCl buffer (pH 7.4) to form micelles, and then NBD spontaneously released was measured at 37 °C in the presence of 10 μM $hGH_{8–13}$ or 10 μM $hGH_{9–13}$ in a Hitachi fluorescent spectrophotometer model F-4500 (excitation wavelength, 470 nM; emission wavelength, 540 nm). The results are the mean (±S.E.) of three determinations.

**Table 2.** Affinity of rat white adipocytes for insulin

| hGH peptide | $K_m$ (M)[a] | Ratio to control |
|---|---|---|
| None (control) | $2.684\times10^{-11}$ | 1.00 |
| $hGH_{8–13}$ (30 μM) | $1.099\times10^{-12}$ | 24.42 |
| $hGH_{9–13}$ (30 μM) | $2.946\times10^{-11}$ | 0.91 |

[a]In the presence of $hGH_{8–13}$ or $hGH_{9–13}$, the $K_m$ values were determined by Lineweaver–Burk plots from the values of Fig. 4A.

6). The RNA samples were subjected to agarose gel electrophoresis and transferred onto nitrocellulose membranes. As shown in Figure 5A, the transcription of HK I was observed in all culture conditions (1–6), and each intensity was the same. The signal of HK II was detected at the same level in conditions 1–6 (Fig. 5B). Then, $hGH_{8–13}$ did not affect the transcript levels of HK I and HK II. Neither HK III nor HK IV gave a signal in all culture conditions (data not shown). Figure 5C shows the control experiments indicating the RNA integrity.

### Analysis of transcript levels of GLUT isoforms in adipocytes

We examined the level of the transcripts encoding four GLUT isoforms in RNA samples obtained from various culture conditions as in the analysis of HK isozymes. Northern blot analysis was performed with 10 μg of RNA. Each intensity of GLUT 1 isoform band was the same in the culture conditions 1–6 (Fig. 6A). GLUT 4 isoform transcript was detected at the same level in all culture conditions (Fig. 6B). Then $hGH_{8–13}$ had no effect on the transcription of GLUT 1 and GLUT 4. No bands of GLUT 2 and GLUT 3 were detected in all culture conditions, as with HK III and HK IV (data not shown). Figure 6C shows the integrity of each RNA sample.

## Discussion

In the present study, we analyzed the structural common features of insulin-potentiating fragments of human growth hormone by computative simulations. From the molecular mechanics (MM) computation and the molecular dynamics (MD) simulation, the macrocyclic structure with $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ sequence in the growth hormonal peptides and its intramolecular atmosphere were found as the essential features to enhance the insulin action (Fig. 1). Indeed the peptides $hGH_{6–10}$, $hGH_{9–13}$ and $hGH_{10–13}$ had no macrocyclic structure in their molecules (Fig. 2A, B and C), and these peptides did not promote the insulin action (Fig. 4A, C and Table 1). Although the β-$Ala^{13}hGH_{1–15}$ formed the macrocyclic structure with $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ (Fig. 2D), it had no effect on the insulin action.[8]The reason for this was though to be that the elongation of the $hGH_{1–15}$ peptide by the replacement of $Ala^{13}$ to a

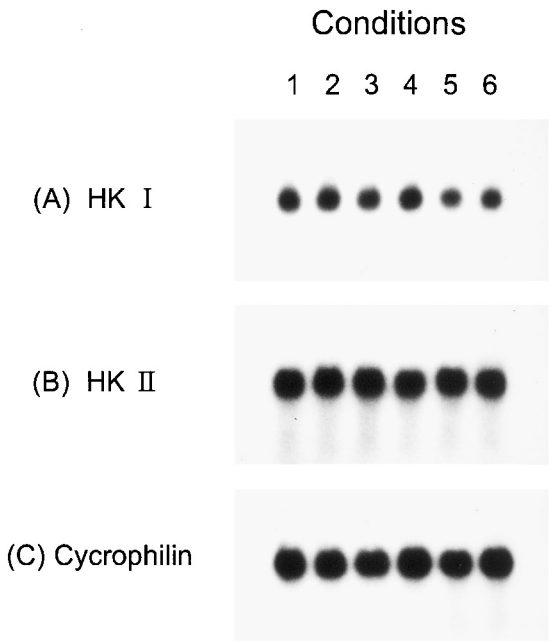


**Figure 5.** Effect of $hGH_{8-13}$ on transcript levels of HK isozymes in white adipocytes. Samples of 10 μg of RNA obtained from rat white adipocytes, which were incubated as follows: without (condition 1) or with $1.22\times10^{-11}$ M insulin (condition 2), 2 nM isoproterenol (condition 3), $1.22\times10^{-11}$ M insulin and 2 nM isoproterenol (condition 4), $1.22\times10^{-11}$ M insulin and 2 nM isoproterenol and 20 μM $hGH_{8-13}$ (condition 5), 20 μM $hGH_{8-13}$ (condition 6) at 37 °C for 2 h, and were subjected to agarose gel electrophoresis, transferred on nitrocellulose membranes and hybridized with the probes for HK I (A), HK II (B) and cycrophilin (C).

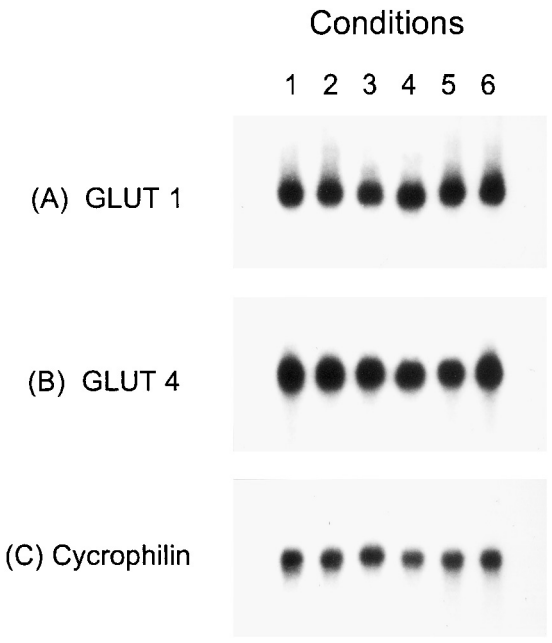


**Figure 6.** Analysis of transcript levels of GLUT isoforms in adipocytes. Samples of 10 μg of RNA obtained from adipocytes as described in the legend for Figure 5 were hybridized with the probes for GLUT 1 (A), GLUT 4 (B) and cycrophilin (C).

β-alanyl group increased the degree of freedom of $Leu^{15}$ carboxyl group (Fig. 1A versus Fig. 2D). Then the carboxylate anion of $Leu^{15}$ in β-$Ala^{13}hGH_{1-15}$ easily approached its macrocyclic $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ region, especially the guanidyl residue in $Arg^8$ (Fig. 2D). On the contrary, the carboxylate anion of $Leu^{15}$ in $hGH_{1-15}$ hardly approached the macrocyclic region, because $Leu^{15}$ carboxyl group was vertically positioned against the macrocyclic region (Fig. 1A). From the energy profiles of the β-$Ala^{13}hGH_{1-15}$ and the $hGH_{1-15}$ through their holding processes, the β-$Ala^{13}hGH_{1-15}$ (Fig. 3A) molecule should be more flexible than $hGH_{1-15}$ (Fig. 3B) and then C-terminal carboxyl group of $Leu^{15}$ easily accessed $Arg^8$ and inhibited the ionic bond formation between $Arg^8$ and $Asp^{11}$ in β-$Ala^{13}hGH_{1-15}$. From these results, we considered that $Ala^{13}$ managed the $Leu^{15}$ movement, and then $hGH_{8-13}$ was an essential unit for the regulation of insulin action. Indeed $hGH_{8-13}$ dose-dependently suppressed insulin-involved fatty acid synthesis in rat white adipocytes (Fig. 4A and B), while $hGH_{9-13}$ had no effect on this insulin action (Fig. 4A and C).

Recognizing the target cells is the first step of expression of the hormonal action, in vivo. It is easily considered that the conditions of cell membranes affect the sensitivity of target cells to hormones, because the target receptors are integrated in the cell membranes.[9–13] Biscoclaurine alkaloids (cepharanthine, tetrandrine, isotetrandrine) from *Stephania cepharantha*[29] are known as agents that stabilize the cell membranes by affecting the fluidity of their lipid bilayer and that cause metabolic changes in several types of cells, and these alkaloids have macrocyclic structures.[30–32] We had been examining the effect of these alkaloids both on the membrane stability and the hormonal action (e.g., insulin-involved fatty acid synthesis), and we obtained the results that these alkaloids stabilized the $C_6$-NBD-PC model membrane and enhanced the insulin action in rat white adipose tissue such as $hGH_{8-13}$ (data not shown, submitted for publication). The macrocyclic structures of these alkaloids are elliptic shaped, and the major- and minor-axes of cepharanthine molecule are 7.8 and 3.6 angstrom, respectively. Similarly, each hGH peptide such as $hGH_{1-15}$, $hGH_{6-13}$, $hGH_{7-13}$ and $hGH_{8-13}$ has an elliptic shaped macrocyclic structure, and the major- and minor-axes of $hGH_{8-13}$ are 7.9 and 3.5 angstrom, respectively. Thus, size of the intramolecular macrocyclic structure of each alkaloid is almost equal to that of the macrocyclic structure in hGH peptides ($hGH_{1-15}$, $hGH_{6-13}$, $hGH_{7-13}$ and $hGH_{8-13}$) compared to $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$. In the present study, $hGH_{8-13}$ increased the stability of the $C_6$-NBD-PC model membrane as these alkaloids, and promoted the insulin-involved fatty acid synthesis (Fig. 4). On the other hand, $hGH_{9-13}$ had no effect both on the membrane stability and the insulin action (Fig. 4C and D). Judging from these results, we considered that the $Arg^8$-$Leu^9$-$Phe^{10}$-$Asp^{11}$ macrocyclic structure in the hGH peptides seems to be responsible for the promotion of insulin action via a modification of the membrane condition.

The uptake of glucose and its phosphorylation are major processes in cellular insulin-involved glucose metabolism. In mammalian cells, four isoforms of the glucose transporter (GLUT 1–GLUT 4) and four isozymes of hexokinase (HK I–HK IV) are important for the

sequential reactions in the glucose metabolism.[14–20] In the previous reports, the mRNA expression of HK isozymes and GLUT isoforms had been examined with 100 nM of insulin, and the enhancement of HK I, II expression and GLUT 1, 4 translation with insulin were reported.[33–38] In the present study, insulin-involved fatty acid synthesis was enhanced by $hGH_{8-13}$ with $10^{-11}$ M-order of insulin (Fig. 4), but the expression of HK I, HK II isozymes and GLUT 1, GLUT 4 isoforms was not affected by $hGH_{8-13}$ with insulin ($1.22\times10^{-11}$ M) in white adipocytes (Figs 5 and 6). Then we considered that the modification of insulin action by the $hGH_{8-13}$ peptide should not be mediated by HK I, II and GLUT 1, 4 isoforms. In rat tissues, the signals of HK III and GLUT 3 were weakly detected only in brain, and the expression of HK IV and GLUT 2 were observed only in liver.[39] We think that HK III, IV and GLUT 2, 3 are not concerned with the insulin action at $10^{-11}$ M in rat white adipocytes. Indeed, we did not detect HK III, IV isozymes and GLUT 2, 3 isoforms in rat white adipocytes which were activated by insulin in the presence of $hGH_{8-13}$.

When an exogenous peptidal product or a chemical compound is found to influence a biological system, it is logical to ask if these molecules are mimicking an endogenous substance. $hGH_{8-13}$ enhanced the insulin action but did not act by itself, then $hGH_{8-13}$ did not mimic an endogenous substance (e.g., insulin). Biscoclaurine alkaloids (cepharanthine, tetrandrine, isotetrandrine) also modified the insulin action but they had no insulin-like activity by themselves (data not shown). From these facts, we considered that $hGH_{8-13}$ and the alkaloids are good lead compounds to design a new type of anti-diabetes drug. Therefore, we are now calculating the disposition of electrostatic potential of hGH peptides (e.g., $hGH_{8-13}$) and biscoclaurine alkaloids (e.g., cepharanthine) to analyze the role of intramolecular macrocyclic structure in the modification of insulin action. Moreover, to regulate the insulin-involved biological action via a modification of the sensitivity of receptor protein to insulin, the analysis of the relationship between the membrane conditions (fluidity, stability) and the insulin activity is currently proceeding.

## Experimental

**Reagents.** Reagents used were as follows: type II collagenase, Sigma, USA; insulin, Novo Nordic, Denmark; 9-anthryldiazomethane (ADAM) and 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphatidylcholine ($C_6$-NBD-PC), Funakoshi, Japan; $hGH_{8-13}$ and $hGH_{9-13}$, Peptide Institute, Japan; Isogen, Nippon Gene, Japan; *Taq* DNA polymerase and *Bca*BEST DNA labeling kit, Takara Shuzo, Japan; [α-$^{32}$P]dCTP (specific radioactivity, 111 TBD/mmol), Amersham Pharmacy Biotech, UK.

**Computative analysis.** Molecular coordinates of human growth hormone in the complex with its receptor protein were obtained from the crystallographic structure, entry name 3HHR from Protein Data Bank, Brookhaven National Laboratory.[21] A molecular modeling of hGH peptides by the molecular mechanics (MM) computation and the molecular dynamics (MD) simulation were performed with Insight II-Discover (CVFF forcefield) (Molecular Simulations Inc., San Diego, USA) on a Silicon Graphics Indigo 2 Impact 10000 Workstation, and the global minimum was searched with CONFLEX (Professor E. Osawa, and Dr. H. Goto, Toyohashi University of Technology, Japan). An isothermal vibration was simulated with the geometry of the molecules protonated at pH 7.4 under the temperature of 300 K. The 3-D-projected view of the optimized structure was drawn by ORTEP-III (Dr. L. J. Farrugia, University of Glasgow, 1996).

**Preparation of adipocytes and lipolysis.** White adipose tissue (WAT; Epididymal fat tissue) was excised from 8 week old male Wistar rats, and adipocytes were prepared from the tissue by type II collagenase digestion as previously described.[40] Adipocytes were washed 3 times with 5 mL of Krebs–Ringer Hepes buffer (KRH; 12 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 12 mM $NaHCO_3$ and 30 mM Hepes, pH 7.4). The cells were collected and suspended in KRH containing 10 mM glucose and 0.1% bovine serum albumin (incubation medium) at a density of $1\times10^7$ cells/mL. Cells ($1\times10^6$ cells) were incubated at 37 °C for 2 h with indicated amounts of each hGH peptide and insulin in the presence of 2 nM isoproterenol in 200 μL of the incubation medium. Free fatty acids released from the cells were measured according to the method of Dole.[41]

**Hydrolysis of a fluorescent phospholipid substrate.** NBD-labeled phosphatidylcholine ($C_6$-NBD-PC) was suspended at $1.29\times10^5$ M in 0.1 M Tris–HCl buffer (pH 7.4) and formed micelles. Fluorescence measurement was performed at 37 °C in the presence of 10 μM $hGH_{8-13}$ or 10 μM $hGH_{9-13}$ peptide in a Hitachi Fluorescent Spectrophotometer Model F-4500 (excitation wavelength, 470 nm; emission wavelength, 540 nm).

**cDNA probes.** All cDNA fragments (HK I to IV and GLUT 1 to 4) were gifts from Dr. Yasuo Shinohara, University of Tokushima, Japan.

**Preparation of RNA samples from adipocytes.** Adipocytes ($5.0\times10^6$ cells) were incubated at 37 °C for 2 h with 20 μM of $hGH_{8-13}$ and $1.22\times10^{-11}$ M insulin in the presence of 2 nM isoproterenol in 1.5 mL of the incubation medium. After incubation period, total RNA was isolated from the adipocytes using Isogen by the method recommended by the supplier, which was essentially based on the report of Chomczynski and Sacchi.[42]

**Northern blotting and determination of transcript levels.** Northern blotting was carried out essentially as described previously.[39] Briefly, RNA was subjected to the electrophoresis in 1.0% agarose gel containing formaldehyde, transferred to a nitrocellulose membrane, and hybridized according to the standard procedure. Each 10 μg of total RNA sample was used for the analysis.

After hybridization with the cDNA probes, the membranes were washed three times with 2 × saline sodium citrate (SSC) containing 0.1% SDS at room temperature for 10 min and twice with 1×SSC containing 0.1% SDS at 60 °C for 30 min each time and exposed to X-ray film on an intensifying screen at −80 °C.

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