ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10): 939-943
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B9-serine residue is crucial for insulin actions in glucose metabolism[1]

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KEY WORDS insulin; adipocytes; monosaccharide transport proteins; phosphorylation

ABSTRACT

AIM: To explore the importance of B9 and B10 amino acid residues in the insulin molecule. **METHODS**: The [B9Glu, B10Asp]insulin (E,D-insulin) receptor binding activity, glucose uptake activity, and lipogenesis activity were measured in isolated adipocytes. The translocation of glucose transporter 4 (Glut4) and the phosphorylation of insulin receptor (IR) stimulated by E, D-insulin were determined by Western blotting. **RESULTS**: Compared with native insulin, the receptor binding activity of E, D-insulin was 31 %; the stimulating activity of E, D-insulin in glucose transport and lipogenesis were 45 % and 40 % respectively; the stimulations of Glut4 translocation and insulin receptor autophosphorylation of E, D-insulin were about 58 % and 46 % respectively. **CONCLUSION**: B9-serine residue is crucial for insulin actions in glucose metabolism.

INTRODUCTION

Insulin is a protein hormone for regulating metabolism, growth, and differentiation of cells. The biological actions of insulin are initiated by insulin binding to its receptors located at target cell surface. X-ray analysis of insulin crystal indicates that B9Ser is a part of the polar B chain N-terminal helical surface, which forms the hydrophilic central core of the hexamer composed of three dimers[1] and B10His located at the surface of the insulin monomer plays a key role in the formation of insulin hexamer[2]. From the study of structure-function relationship of insulin, it was known that mutation of B9Ser to B9Asp[3] or B9Glu[4] blocked the dimer formation mainly because of the intermolecular repulsive action of the negative charge of B9Asp or B9Glu residue and its biological activity *in vivo* was found to be 40 % compared with native insulin. It was also reported that [B10Asp]insulin possessed very high activity *in vitro*, approximately 435 % compared with native insulin, and still formed dimer[5]. Burke and his colleagues[6] used chemical methods to obtain superactive insulin analogues, in which not only B10His was substituted by Asp, but other positions were also modified. In order to investigate the cooperative effect of B9 and B10 residues on the structure and function of insulin, we prepared E,D-insulin by site-directed mutagenesis *in vitro* and found that the E, D-insulin exhibited biological activity nearly as high as porcine insulin *in vivo*, but its receptor binding ability on human placental membrane was determined to be only 34.4 % of porcine insulin[7]. The study on the structure and function of insulin is also useful to find insulin analogues with clinical applications. As we know, native insulin is prone to form dimer and hexamer while only monomer has physiological actions, so injected insulin preparations require time to dissociate into monomer. Therefore, we need to find monomeric insulin unable to form dimer or hexamer that will become a candidate of insulin preparations with rapid onset and short duration of action. It was also observed in our laboratory that E, D-insulin was monomer in solution. In this study, we examined receptor binding capacity of E,D-insulin and its effect on glucose metabolism in rat adipocytes.

MATERIALS AND METHODS

Materials Crystalline porcine insulin was purchased from Nanjing Biochemical Pharmaceutical Factory (Nanjing, China). [B9Glu, B10Asp]insulin (E, D-insulin) prepared by site-directed mutagenesis was

[1] Project supported by Chinese 863 High Technology Foundation, No 863-103-01-01.
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Received 2001-04-19 Accepted 2001-07-05

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

homogenous in PAGE and HPLC[7]. *D*-[3-^{3}H]-glucose, enhanced chemiluminescence (ECL) and anti-phosphotyrosine-HRP antibody (anti-pTyr-HRP) were purchased from Amersham. Bovine serum albumin (BSA, Fraction V, RIA grade) and collagenase (type IV) were from Sigma and Worthington Biochemical Corporation respectively. Polyclonal Glut4 antibody was prepared in our laboratory using synthetic C-terminal tetradecapeptide of glucose transporter 4 (Glut4). The goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) was a kind gift from Professor Zhang Zu-Chuan (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China). Other reagents were of AR grade. Male Sprague-Dawley rats (Grade Ⅱ, Certificate № 005) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences.

Preparation of isolated adipocytes The isolated adipocytes were prepared from the whole epididymal fat pads of male Sprague-Dawley rats (190 g ± 10 g) using a modification of Rodbell's method[8,9]. Briefly, the whole epididymal fat pads were collected from 8 rats, then minced and digested at 37 ℃ with 16 mg crude collegenase in 16 mL of KRPH buffer (Krebs-Ringer buffer with sodium phosphate 1 mmol·L^{-1} and HEPES 12.5 mmol·L^{-1}) containing BSA 5 % and glucose 0.55 mmol·L^{-1} at pH 7.4. After 40 – 60 min digestion, the liberated cells were collected, washed five times and then resuspended in the same buffer.

Treatment of adipocytes with insulins and preparation of membranes The cells were resuspended in 6 mL of KRPH buffer containing 1 % bacitracin, 2 % BSA, and insulin 3.4 nmol·L^{-1} or E,D-insulin 3.4 nmol·L^{-1} in 50 mL polypropylene tubes and incubated at 37 ℃ with gentle shaking for 15 min and 5 min for Glut4 and phosphorylation experiment respectively. The cells were washed three times with HEPES 20 mmol·L^{-1}/edetic acid 1 mmol·L^{-1}/sucrose buffer 255 mmol·L^{-1}, pH 7.4, and then resuspended in 10 mL of the same buffer, homogenized with 10 strokes in a 55 mL Teflon Pesple homogenizer at 20 ℃. Subcellular fractions were isolated by centrifugation at 16 000 × *g* for 15 min at 4 ℃ using a modification of the method described by Mckeel and Jarett[10]. The fat cake and the supernatant were carefully removed, the pellet was resuspended, centrifuged and resuspended in 0.2 mL of the same buffer. The suspension was stored at – 20 ℃ until use.

Protein concentration The protein concentration of membrane preparations was determined using Bradford assay with BSA as standard[11].

Receptor binding assay The method used in our laboratory[12] was slightly modified. Adipocytes were incubated with ^{125}I-insulin (1850 Bq) and increasing concentrations of unlabelled insulin or E, D-insulin in a final volume of 0.5 mL KRPH buffer containing 5 % BSA at 37 ℃ for 60 min. The reactions were terminated by dilution with 2.5 mL of PBS buffer followed by adding 1 mL of dinony phthalate and then the tubes were centrifuged at 2860 × *g* at 4 ℃ for 10 min. The cells in the upper layer were transferred to γ-counter tubes and the radioactivity was measured. The nonspecific binding was determined in the presence of an excess of unlabelled insulin and the specific binding was calculated by subtracting the nonspecific binding from the total binding. All assays were performed in triplicates.

Glucose uptake Glucose uptake was carried out by the method used in our laboratory[13] with minor modification. Briefly, adipocytes were incubated with *D*-[3-^{3}H]-glucose 20.9 nmol·L^{-1} and different concentrations of the sample in a final volume of 0.5 mL KRPH containing 5 % BSA for 60 min at 37 ℃ with gentle shaking. After dilution with 3 mL of saline solution and addition of 0.5 mL of dinony phthalate, the tubes were centrifuged and the cells were transferred to a vial containing 2 mL of scintillation solution and counted.

Lipogenesis The experiment was performed according to the method described by Moody *et al*[14]. Adipocytes together with 0.19 nBq *D*-[3-^{3}H]-glucose and indicated concentrations of the sample in 0.5 mL of KRPH buffer containing 1 % BSA and glucose 0.55 mmol·L^{-1} were incubated at 37 ℃ with gentle shaking for 120 min, and then 3 mL of scintillation solution was added, mixed, and counted.

Immunoblot analysis Equal amounts of membrane proteins were subjected to 10 % SDS-PAGE under reducing conditions as previously reported[15] and transferred to nitrocellose membrane. The Glut4 and phosphorylated IR β-subunit were immunodetected by polyclonal rabbit anti-Glut4 antibody and anti-pTry-HRP respectively, and the blots were developed by enhanced chemiluminescence method, using horseradish peroxidase-conjugated goat anti-rabbit IgG as second antibody to detect Glut4. The quantitation of the relative band intensity was performed by laser scanning densitometry.

RESULTS

Inhibition of ^{125}I-insulin binding to rat adi-

pocytes Specific binding of ^{125}I-insulin to adipocytes was inhibited by unlabelled insulin and E, D-insulin in a concentration-dependent manner (Fig 1). The sample concentrations at 50 % displacement (IC_{50}) were 3.2 nmol · L^{-1} for E, D-insulin and 0.99 nmol · L^{-1} for porcine insulin respectively, ie, the potency of E, D-insulin was only 31 % compared with native insulin.

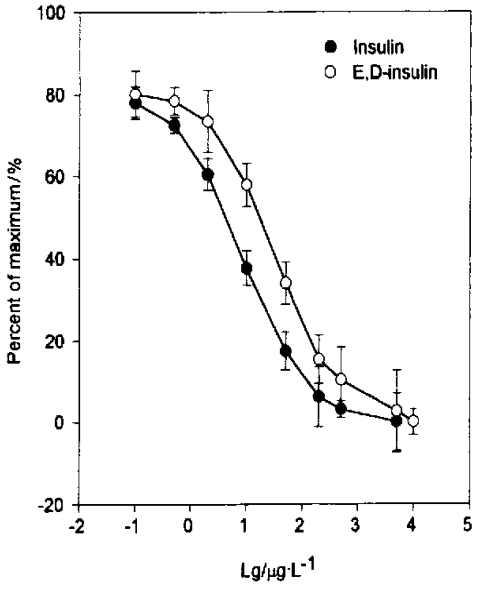

Fig 1. Displacement curve of E, D-insulin in the binding of ^{125}I-insulin to adipocytes. n = 3 experiments. $\bar{x} \pm s$.

Glucose uptake and lipogenesis stimulated by E, D-insulin The activities of E, D-insulin in stimulating glucose uptake and lipogenesis in rat adipocytes were measured and compared with native insulin (Fig 2). The sample concentrations at half maximal stimulation (EC_{50}) of E, D-insulin were 0.22 nmol/L for glucose uptake and 0.35 nmol · L^{-1} for lipogenesis, while those of porcine insulin were 0.10 nmol · L^{-1} and 0.14 nmol · L^{-1} respectively. So, the potency of E, D-insulin was calculated to be about 40 % of porcine insulin.

Glut4 translocation and IR autophosphorylation stimulated by mutant insulin The binding of insulin to IR α-subunit results in the autophosphorylation of IR β-subunit[16] and the translocation of glucose transporters[17,18]. Among the seven known facilitative glucose transporters (Glut 1 to 7)[19], Glut4, which is sensitive to insulin for its translocation from an intracellular membrane compartment to the plasma membrane,

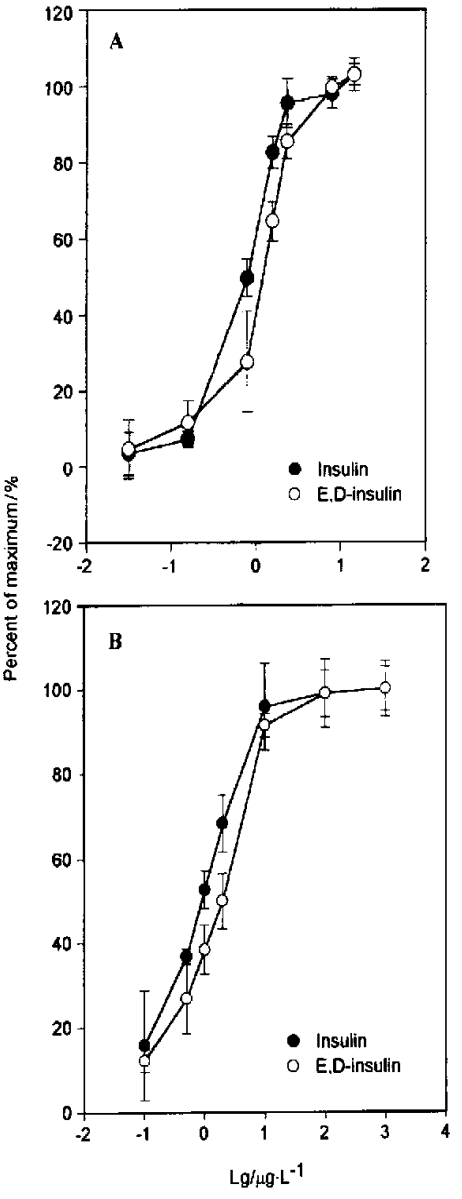

Fig 2. Effect of insulin and E, D-insulin on *D*-[3-^{3}H]-glucose uptake (A) and lipogenesis (B) in rat adipocytes. n = 3 experiments. $\bar{x} \pm s$.

is particularly important in regulating glucose uptake in adipocytes[20]. Therefore, we measured the Glut4 content at the plasma membrane and the autophosphorylation of IR β-subunit in adipocytes after insulin or E, D-insulin treatment. The immunoblotting analysis showed that the ability of E, D-insulin to stimulate Glut4 translocation was 58 % and 46 % for stimulating IR β-subunit autophosphorylation compared with porcine insulin (Fig 3).

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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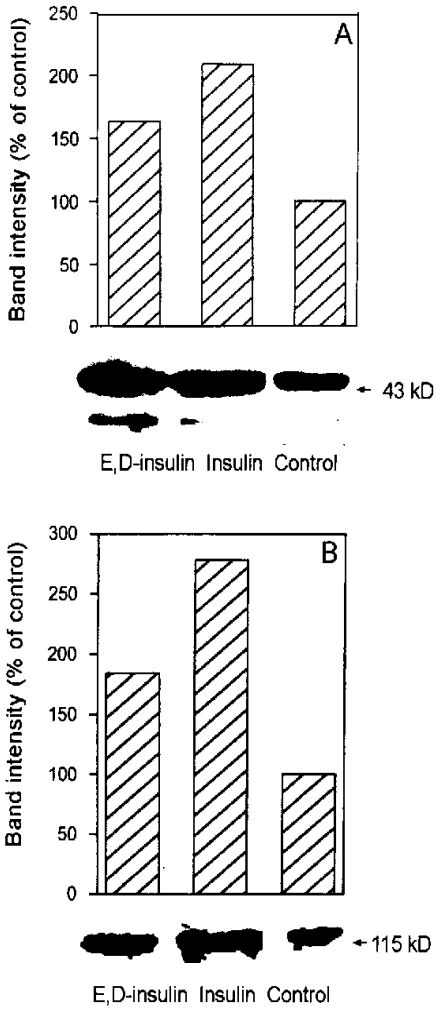

Fig 3. Effect of E, D-insulin and insulin on Glut4 translocation at plasma membrane (A) and phosphorylation of IR β-subunit (B) in rat adipocytes. The results represent one typical example of three experiments.

DISCUSSION

Receptor binding, glucose uptake, lipolysis, lipogenesis, and the translocation of Glut4 in adipocytes are usually used to determine the *in vitro* biological activity of insulin mutants and analogues. In this paper, we reported the receptor binding activity and *in vitro* activities of E, D-insulin in rat adipocytes system. It was shown that E, D-insulin exhibited 31 % receptor binding activity compared with porcine insulin in rat adipocytes, which was similar to the results obtained in human placental membrane[7]. However, the *in vitro* biological activities determined in adipocytes were found to be much lower than that of porcine insulin and disagreed with the full *in vivo* activity[7]. This unparalleled relationship of activities *in vivo* and *in vitro* was also observed in the study on other insulin analogues, showing that other factors may affect *in vivo* activity of insulin. Therefore, it is necessary to test *in vitro* biological activity in order to understand directly the structure and function relationship of insulin. It was shown that the substitution of B9Ser in [B10Asp]insulin with superactivity *in vitro* decreased markedly its activities to about 10 %, indicating that B9Ser is vital to the activity of insulin.

ACKNOWLEDGEMENTS We would like to thank Prof ZHANG Xin-Tang and Ms YAO Shi-Yin for their kind technical assistance, and Prof ZHANG Zu-Chuan for his generous offering of goat anti-rabbit antibody.

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B9位丝氨酸残基是胰岛素的葡萄糖代谢作用所必需的[1]

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关键词 胰岛素；脂肪细胞；单糖转运蛋白类；磷酸化

目的：研究B9和B10位氨基酸残基在胰岛素分子中的重要性. **方法**：在分离的大鼠脂肪细胞中测定[B9Glu, B10Asp]胰岛素的受体结合活性、葡萄糖吸收活性和脂生成活性. 用Western印迹法检测[B9Glu, B10Asp]胰岛素对葡萄糖转运蛋白-4(Glut4)转位和胰岛素受体自磷酸化的刺激活性. **结果**：[B9Glu, B10Asp]胰岛素的受体结合活性和刺激葡萄糖吸收、脂生成、Glut4转位和胰岛素自磷酸化活性分别是天然胰岛素的31 %、45 %、40 %、58 %和46 %. **结论**：B9位丝氨酸残基是胰岛素的葡萄糖代谢作用所必需的.

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