A new recombinant pituitary adenylate cyclase-activating peptide-derived peptide efficiently promotes glucose uptake and glucose-dependent insulin secretion

Yi Ma, Tianjie Luo, Wenna Xu, Zulu Ye, and An Hong*

Department of Cell Biology, Institute of Biological Medicine, Jinan University, Guangzhou 510632, China
*Correspondence address. Tel: +86-20-85223266; Fax: +86-20-85221983; E-mail: makesi8866@163.com

The recombinant peptide, DBAYL, a promising therapeutic peptide for type 2 diabetes, is a new, potent, and highly selective agonist for VPAC2 generated through site-directed mutagenesis based on sequence alignments of pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal peptide (VIP), and related analogs. The recombinant DBAYL was used to evaluate its effect and mechanism in blood glucose metabolism and utilization. As much as 28.9 mg recombinant DBAYL peptide with purity over 98% can be obtained from 1 l of Luria-Bertani medium culture by the method established in this study and the prepared DBAYL with four mutations (N10Q, V18L, N29Q, and M added to the N-terminal) were much more stable than BAY55-9837. The half-life of recombinant DBAYL was about 25 folds compared with that of BAY55-9837 in vitro. The bioactivity assay of DBAYL showed that it displaced [125I]PACAP38 and [125I]VIP from VPAC2 with a half-maximal inhibitory concentration of 48.4 ± 6.9 and 47.1 ± 4.9 nM, respectively, which were significantly lower than that of BAY55-9837, one established VPAC2 agonists. DBAYL enhances the cAMP accumulation in CHO cells expressing human VPAC2 with a half-maximal stimulatory concentration (EC50) of 0.68 nM, whereas the receptor potency of DBAYL at human VPAC1 (EC50 of 737 nM) was only 1/1083 of that at human VPAC2, and DBAYL had no activity toward human PAC1 receptor. Western blot analysis of the key proteins of insulin receptor signaling pathway: insulin receptor substrate 1 (IRS-1) and glucose transporter 4 (GLUT4) indicated that the DBAYL could significantly induce the insulin-stimulated IRS-1 and GLUT4 expression more efficiently than BAY55-9837 and VIP in adipocytes. Compared with BAY55-9837 and PACAP38, the recombinant peptide DBAYL can more efficiently promote insulin release and decrease plasma glucose level in Institute of Cancer Research (ICR) mice. These results suggested that DBAYL could efficiently improve glucose uptake and glucose-dependent insulin secretion by VPAC2-mediated effect.

Keywords pituitary adenylate cyclase-activating peptide; type 2 diabetes; insulin; VPAC2-mediated effect; recombinant peptide

Received: June 29, 2012 Accepted: August 6, 2012

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the superfamily of metabolic, neuroendocrine, and neurotransmitter peptide hormones and belongs to the secretin, glucagons, and vasoactive intestinal peptide (VIP) family [1,2]. PACAP exists as either a 38-amino acid (PACAP38) or 27-amino acid (PACAP27) peptide. PACAP27 corresponds to the N-terminal 27-amino acid portion of PACAP38 and exhibits the same biological activity as PACAP38 [3,4]. The action of PACAP is mediated through three G protein-coupled receptors, PAC1, VPAC1, and VPAC2. PAC1 receptor exhibits high affinity for PACAP38 and PACAP27, but much lower affinity for VIP. VPAC1 and VPAC2 receptors exhibit high affinity for PACAP38 and PACAP27, but much lower affinity for VIP. VPAC1 and VPAC2 receptors exhibit similar high affinity for PACAP38, PACAP27, and VIP [5]. PACAP is widely distributed in the brain and peripheral organs, notably in the endocrine pancreas, gonads, respiratory, and urogenital tracts, which has been shown to have effects on many pathological states including Parkinson’s disease [6], diabetes [7,8], ischemia [9], traumatic injury [10], immunological disorders [11,12], myeloma kidney injury, and so on [13]. Most of these neuroprotective actions of PACAP are mediated through the selective PAC1 receptor whereas the effects on peripheral organs often involve VPAC1 or VPAC2 receptor. PACAP has been shown to increase insulin secretion from the pancreas through VPAC2 receptor [14,15]. But the role of PACAP in the control of glucose homeostasis is complex, because it also plays a role in increasing glucagon and catecholamine secretion, which increases glucose output from the liver through VPAC1-mediated effect [16]. Therefore, PACAP derivative as
VPAC2-specific agonist, which would stimulate glucose-dependent insulin secretion from pancreatic β-cell without leading to increased glucose production by the liver could be used for clinical treatment of type 2 diabetes. Development of BAY55-9837, an established highly selective VPAC2 agonist, as a potential peptide therapeutic for the treatment of type 2 diabetes was limited by its poor peptide stability [14]. To overcome the limitation, the recombinant peptide DBAYL with 32 amino acids was designed and generated through site-directed mutagenesis by gene-recombination technology. The recombinant DBAYL (N10Q, V18L, N29Q, and M added to the N-terminal) were much more stable than BAY55-9837. DBAYL enhances the cAMP accumulation in VPAC2-CHO cells with higher bioactivity than BAY55-9837. DBAYL could more efficiently induce the expression of the key proteins of insulin receptor signaling pathway including insulin receptor substrate 1 (IRS1) and glucose transporter 4 (GLUT4) than BAY55-9837 in adipocytes [17,18]. In addition, DBAYL treatment increased the insulin-stimulated GLUT4 translocation to the plasma membrane. Corresponding to these results, glucose uptake activity of differentiated 3T3-L1 adipocytes treated with DBAYL were significantly improved, which was better than BAY55-9837.

Thus, insulin signal transduction was more efficiently improved by DBAYL through VPAC2-mediated effect. DBAYL, a novel recombinant PACAP-derived peptide, as highly selective agonist for VPAC2, can hopefully be a peptide therapeutic for type 2 diabetes through efficiently promoting glucose uptake and glucose-dependent insulin secretion.

Materials and Methods

Materials

Chitin beads and the plasmid pKYB-MCS were purchased from New England Biolabs (NEB, Ipswich, USA). Escherichia coli strain ER2566 was kept in our laboratory.

The restriction enzymes were purchased from New England Biolabs. T4 DNA ligase was obtained from TaKaRa (Dalian, China). Synthetic peptides were purchased from Sinoasis Pharmaceuticals (Guangzhou, China). Primer synthesis and DNA sequencing were performed by Invitrogen Company, Guangzhou Branch (Guangzhou, China). VPAC2-CHO cell line was constructed in our laboratory. 3T3-L1 adipocytes were provided by Dr Zhang WJ (College of Life Sciences, Wuhan University, Wuhan, China).

Construction and identification of the expression plasmid pKY-DBAYL

The DBAYL gene was designed according to the bias of E. coli for the codons to ensure its high expression. The gene was synthesized and amplified in two steps as described previously [7] using three oligonucleotides primers: F1: 5′-GGTGATCATATGCTAGTACGATGCAGGT GTTACCCGATCATGATACCCGTCGTTA-3′, containing an Ndel site (underlined); F2: 5′-CATATT TTTCCGCCGCACTGTTTCGACACGGGT-3′; F3: 5′-CCACCAGCTCTTCCGCAATAACGTTTCTGTTAA TGCTCTGCAGATATTATTTT-3′, containing an SapI site (underlined); GTGCGT at the 5′ end of F3 and CCACCA at the 5′ end of F2 are the protecting bases. After polymerase chain reaction (PCR) products were purified by the PCR clean-up kit (Qiagen, Hilden, Germany) and digested with Ndel and SapI, the DNA fragment was directly ligated to a gel-purified Ndel/SapI digested pKYB-MCS vector (NEB) to yield the expression plasmid pKY-DBAYL. pKY-DBAYL containing DBAYL gene was confirmed by DNA sequencing using the T7 promoter as the sequencing primer (Fig. 1).

Expression of fusion protein

The recombinant expression vector pKY-DBAYL was transformed into the E. coli strain ER2566 with the optimized procedure [19]. Briefly, the cells were grown at 37°C to a density of OD$_{660}$ = 0.8 and induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.5 mM.
The induced cells were incubated for 6 h at 35°C and collected by centrifugation at 10,621 g for 20 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the expression of the fusion protein. The cell pellet was resuspended in buffer A containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 1 mM EDTA by gentle shaking for 20 min, and then disrupted with JN-3000 PLUS low-temperature ultra-high-pressure continuous flow cell crusher (JNBIO, Guangzhou, China) at the following conditions: diluted bacteria concentration of 18% by buffer A, crushing pressure of 1700 bar and cooling temperature of 3°C. The lysate was then centrifuged at 10,621 g for 30 min at 4°C and the supernatant was subjected to purification and preparation of target peptide by chitin beads affinity chromatography.

**Preparation and identification of the recombinant peptide DBAYL**

The supernatant (1.5 l) was passed through a column (4.5 cm × 20 cm) packed with 25 ml chitin beads at a flow rate of 0.5 ml/min. After the supernatant was loaded on the column, the flow rate was raised to 2 ml/min and the column was thoroughly washed with more than 10 bed volume of buffer A. Then 80 ml of buffer B containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, and 100 mM β-mercaptoethanol was then quickly passed through the column to distribute β-mercaptoethanol evenly throughout the resin and the column flow was stopped. The column was incubated at 25°C for 24 h. Fractions containing DBAYL were obtained by eluting the column with buffer A. Then the recombinant peptide DBAYL was purified and prepared by reverse-phase high-performance liquid chromatography (HPLC) system using 4.6 mm × 150 mm 300 SB-C18 Sep-Pak column (Agilent Technologies, Beijing, China) through gradient elution with increasing concentration of acetonitrile from 2% to 55% for 45 min at 1 ml/min. The eluate containing DBAYL was dried by lyophilization. Prepared DBAYL at a final concentration of 1 mg/ml in 45% acetonitrile containing 0.1% trifluoroacetic acid was analyzed by 4000 Q TRAP electrospray ionization-mass spectrometry (ESI-MS; Applied Biosystems, Foster City, USA). Peptide concentrations were determined by comparing the OD_{280} values of peptide stock solutions in the assay buffer with the predicted extinction coefficient [20].

**Stability assay**

DBAYL, BAY55-9837, PACAP38, or VIP at a final concentration of 1 mg/ml in 20 mM sodium phosphate buffer (pH 8.0) containing 150 mM sodium chloride were incubated at 37°C. At different time points, samples were collected and analyzed by liquid chromatography mass spectrometry, a rapid and sensitive method to detect degradation of polypeptide in these formulations. A 2-ml sample was injected into HPLC-ESI-MS system containing 1.0 mm × 150 mm 300 SB-C18 Sep-Pak (Agilent Technologies, Santa Clara, USA) column and analyzed under the condition of increasing concentration of acetonitrile from 2% to 55% for 55 min at 0.05 ml/min by HPLC-ESI-MS system.

**Competition receptor binding assay**

The potential of DBAYL to displace [125I]PACAP38 and [125I]VIP by competitively binding to the human VPAC2 receptor was examined in VPAC2-CHO cell membrane prepared previously[7]. Briefly, 10 mg of membrane was incubated with 0.1 nM [125I]PACAP38 (Phoenix Pharmaceuticals, Mountain View, USA) or [125I]VIP (PerkinElmer Life and Analytical Sciences, Boston, USA) in the presence of increasing concentrations of DBAYL peptide, in a total volume of 100 ml of 20 mM HEPES (pH7.4) containing 150 mM NaCl, 0.5% BSA, 2 mM MgCl₂, and 0.1 mg/ml bacitracin at 37°C. After being incubated for 20 min, the membrane was collected on GF/C filters pretreated with 0.1% polyethylenimine. The filters were washed with 25 mM cold Na₃PO₄ containing 1% BSA and counted on a gamma counter. Non-specific binding was defined as the residual binding in the presence of 1 nM recombinant PACAP38 (i.e. rPACAP38) or VIP and was always <20% of the total binding. The assay of PACAP38, VIP, and BAY55-9837 were taken as the positive controls. [K^{15},R^{16},L^{27}]VIP(1–7)/GRF(8–27), a VPAC1-specific agonist, was used as the negative control in the receptor binding assay [21]. Each assay was performed at least three times.

**Assay of cAMP accumulation induced by DBAYL**

Human PACAP receptor-transfected cells, VPAC1-CHO, VPAC2-CHO, and PAC1-CHO cells, cultured in the Dulbecco’s modified Eagle’s medium at 37°C were scraped off with rubber policeman and washed with PBS twice. The density of the cells was adjusted to 2 × 10⁶ cells/ml. DBAYL or rPACAP38 was added to the 500-ml cell suspension, and the concentrations of the peptide were ranged from 1 × 10⁻¹² to 1 × 10⁻⁵ M. The mixtures were incubated at 37°C for 5 min, then two volumes of 0.2 M HCl was added, and the mixtures were incubated at room temperature for another 20 min. Cells were lysed by pipetting up and down until the suspension was homogeneous. The precipitate was removed by centrifugation at 225 g for 10 min, and the supernatant was transferred into test tube and cAMP concentrations were measured by using the cyclic AMP enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, USA).

**Western blot analysis of IRS-1 and GLUT4 induced by DBAYL**

Cell culture and induction of 3T3-L1 adipocytes were carried out as described previously [22]. Differentiated...
3T3-L1 adipocytes were incubated with 100 nM insulin for 20 min [23]. After being washed twice with PBS buffer, differentiated 3T3-L1 adipocytes were cultured for 48 h in medium, respectively, containing 0 and 1 μM of DBAYL, BAY55-9837 or VIP. Then the total protein was extracted. After the total protein was separated by 12% SDS-PAGE and transferred onto poly(vinylidene difluoride) membranes (Immobilon P; Millipore, Billerica, USA), the membranes were incubated with the IRS-1 rabbit mAb (Cell Signaling Technology, Boston, USA) or anti-GLUT4 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 2 h at room temperature. The horseradish peroxide (HRP)-conjugated goat-anti-rabbit IgG (Immunology Consultants Laboratory, Portland, USA) or sheep-anti-mouse HRP-IgG (BioFX Laboratories, Owings Mills, USA) was used as the second antibody. Protein bands were visualized by using an ECL kit-glucose oxidase method (Applygen Technologies Inc., Beijing, China). The plasma insulin was measured using RIA kit (Linco Research, Charles, USA) in the First Affiliated Hospital of Jinan University (Guangzhou, China).

**Results**

**Expression and preparation for DBAYL**

The fusion proteins consisting of target peptide-, intein- and chitin-binding domain (i.e. DBAYL-intein-CBD) were expressed through a recombinant peptide expression vector, pKY-DBAYL, in *E. coli* strain ER2566. The fusion proteins were purified using chitin affinity column. The cleavage of intein was induced by β-mercaptoethanol and the target peptide, DBAYL was released. Then the recombinant peptide DBAYL was further purified and prepared by reverse phase HPLC system. About 28.9 mg of recombinant DBAYL peptide over 98% of purity can be obtained from 1 l of Luria-Bertani medium. The prepared DBAYL was analyzed and identified by ESI-MS. Figure 2 showed that the molecular weight of DBAYL from ESI-MS was 3916.6 Da, which was consistent with the theoretical value (3916.5 Da). The purity of prepared DBAYL was over 98% by the analytical HPLC determination method.

**Peptide stability improved by site-directed mutagenesis**

The recombinant DBAYL was tested together with BAY55-9837, PACAP38, and VIP for stability at 37°C in 20 mM sodium phosphate buffer (pH 8.0) containing 150 mM sodium chloride. After 4 weeks at 37°C, the main peptide peaks for BAY55-9837, PACAP38, and VIP were remarkably diminished and the slower migrating peak emerged, probably as a result of peptide degradation. On the other hand, DBAYL exhibited dramatic improvement in stability, losing only 7.7% of the main peak. The stability data in 4 weeks showed that the half-life of recombinant DBAYL was about 25 folds compared with that of BAY55-9837 *in vitro*, and the half-life of wild-type PACAP38 and VIP is slightly shorter than the BAY55-9837 *in vitro* (Fig. 3).

**DBAYL selectively binding to VPAC2 receptor**

Human VPAC2 receptor-transfected cells, and VPAC2-CHO cells, were used for competition receptor binding assay. Competition binding of [125I]PACAP38 or [125I]VIP...
membranes purified from CHO cells identified DBAYL as a VPAC2-selective peptide (Fig. 4). DBAYL competitively displaced 
$[125\text{I}]\text{PACAP38}$ from VPAC2, with a half-maximal inhibitory concentration (IC50) of $48.4 \pm 6.9 \text{nM}$, and the IC50 of the recombinant PACAP38, VIP, and BAY55-9837 were $18.1 \pm 5.3$, $21.2 \pm 4.0$, and $68.3 \pm 8.1 \text{nM}$, respectively [Fig. 4(A)]. DBAYL competitively displaced $[125\text{I}]\text{VIP}$ from VPAC2 with an IC50 of $47.1 \pm 4.9 \text{nM}$, and the IC50 for rPACAP38, VIP, and BAY55-9837 at human VPAC2 were $19.7 \pm 4.9$, $18.0 \pm 2.6$, and $70.3 \pm 3.7 \text{nM}$, respectively [Fig. 4(B)]. Whereas the IC50 for VIP(1–7)/GRF(8–27), an established human VPAC1-specific agonist, at human VPAC2 was over $20 \mu\text{M}$. These results showed that DBAYL could competitively displace $[125\text{I}]\text{PACAP38}$ and $[125\text{I}]\text{VIP}$ by binding to human VPAC2 receptor in VPAC2-CHO cells. In two competition receptor-binding experiments, the IC50 of DBAYL was significantly lower than that of BAY55-9837, the established VPAC2-specific agonist.

Receptor potency of DBAYL at PACAP receptor subtypes
The accumulation of cAMP in human PACAP receptor-transfected cells (VPAC1-CHO, VPAC2-CHO, and PAC1-
CHO cells) was used as an index of the agonist activity. DBAYL was a potent agonist for the VPAC2 receptor with a half-maximal stimulatory concentration (EC50) of 0.68 nM. However, the receptor potency of DBAYL at human VPAC1 (EC50 of 737 nM; Fig. 5) was only 1/1083 of that at human VPAC2, and DBAYL had no activity toward human PAC1 receptor.

Three receptors subtypes are both activated by rPACAP38. rPACAP38 was a potent agonist at human PAC1 with an EC50 of 0.57 nM, and the EC50 for rPACAP38 at human VPAC1 and VPAC2 receptor were 0.97 and 0.99 nM, respectively (Fig. 5).

These results showed that DBAYL was a VPAC2-specific agonist with high potency and bioactivity, whereas rPACAP38 could activate human PAC1, VPAC1, and VPAC2 receptor with different affinity.

**In vitro effects of DBAYL on the key proteins in insulin receptor signaling pathway**

The expression levels of IRS1, a key and essential protein for insulin signal transduction and GLUT4, an important rate-limiting factor of the glucose transport, were significantly increased in differentiated 3T3-L1 adipocytes treated with DBAYL.

**Figure 4** Displacement of $[^{125}I]$PACAP38 (A) or $[^{125}I]$VIP (B) by DBAYL, rPACAP38, VIP, BAY55-9837, and VIP(1–7)/GRF(8–27) in membranes purified from CHO cells expressing human VPAC2. The results are expressed as percentage of maximum binding to $[^{125}I]$PACAP38 or $[^{125}I]$VIP.

**Figure 5** Induced cAMP accumulation by DBAYL or PACAP38 in CHO-VPAC2, CHO-VPAC1, and CHO-PAC1 cells Results are expressed as the percentage of maximum cAMP accumulation by PACAP38. Data are the mean of three separate experiments.

**Figure 6(A)** showed that the insulin-stimulated IRS1 and GLUT4 expression levels in differentiated 3T3-L1 adipocytes treated with DBAYL were 3.1 and 2.9 folds, respectively, of that in blank control group without DBAYL treatment. The effects of increasing IRS1 and GLUT4 expression by DBAYL were significantly stronger than VIP and BAY55-9837.

Simultaneously, GLUT4 of translocating to the plasma membrane was significantly increased in differentiated 3T3-L1 adipocytes treated with DBAYL. **Figure 6(B)**
showed that DBAYL treatment and combined treatment of insulin plus DBAYL significantly increased the GLUT4 translocation to the plasma membrane by 51%, which was agreed well with the glucose uptake results. Lower doses of insulin could also increase GLUT4 translocation, and combined treatment of insulin plus DBAYL could more effectively promote GLUT4 translocation to the plasma membrane. As a VPAC2-specific agonist, DBAYL may significantly increase the GLUT4 translocation to the plasma membrane in a non-insulin-dependent manner, and DBAYL had the biological synergistic effect with insulin on GLUT4 translocation.

**DBAYL promoted glucose uptake activity of differentiated 3T3-L1 adipocytes**

Glucose uptake activity of differentiated 3T3-L1 adipocytes treated with different concentrations of DBAYL was all significantly improved in different degrees. Figure 7 showed that 1 and 5 μM of DBAYL increased glucose uptake of differentiated 3T3-L1 adipocytes by 43% and 49%, respectively.

Improvement effect of DBAYL was better than BAY55-9837 at the same concentration. As shown in Fig. 7, 1 and 5 μM of BAY55-9837 increased glucose uptake of differentiated 3T3-L1 adipocytes by 16% and 34%, respectively.

**In vivo effects of DBAYL on insulin release and glucose disposal in ICR mice**

As shown in Table 1, compared with normal saline group, recombinant DBAYL (0.5 μg/kg) obviously promoted the insulin release and decreased the level of plasma glucose after giving glucose by gavage in ICR mice. Furthermore, the results showed that the biological effects of DBAYL were significantly better than BAY55-9837 and rPACAP38. Because of acting on three receptors subtypes, rPACAP38 can not effectively decreased the plasma glucose level of ICR mice after glucose gavage.

**Discussion**

At present, the main approach to treat type 2 diabetes is to maintain euglycemia through administration of sulfonylurea drugs that increase insulin levels or by injecting insulin itself. Both therapies produce significant bouts of hypoglycemia, because their onset of action is independent of the prevailing level of glucose. New therapies that retain or enhance glucose-dependent insulin secretion would be a significant advance, since they would avoid the risk of hypoglycemia.

PACAP could activate both VPAC1 and VPAC2. VPAC2 activation enhances glucose disposal by stimulating insulin secretion while VPAC1 activation elevates hepatic glucose output [26]. Wild-type PACAP could not effectively lower blood sugar in vivo because the increase in glucose production may offset the increase in insulin secretion. Therefore, clinical treatment of diabetes requires a VPAC2-specific agonist that would enhance pancreatic β cell insulin release without causing increased glucose production [27]. VPAC2-specific agonists such as BAY55-9837, Ro25-1553, and hexanoyl-VIP (C6-VIP) produced by chemical synthesis have been demonstrated to induce insulin secretion from β cells in a glucose-dependent manner [28].

In this report, we provide a novel gene recombinant PACAP-derived peptide that is a VPAC2-specific agonist with high stability. Our previous studies had shown that BAY55-9837 and some other polypeptides had potential instability due to either oxidation or deamidation because of several certain amino acid composition [7,19]. Stability analysis showed that the prepared DBAYL with four mutations (N10Q, V18L, N29Q, and M added to the N-terminus) were much more stable than BAY55-9837, wild-type PACAP, and VIP. DBAYL lost only 7.7% of the main peak during the 4-week incubation at 37°C, and the half-life of DBAYL was about 25 folds compared with that of BAY55-9837 in vitro.

Compared with three previously studied VPAC2 agonists, BAY55-9837, hexanoyl-VIP (C6-VIP), and Ro 25-1553 [14,18], or wild-type PACAP and VIP, there was one methionine at the N-terminus of the recombinant DBAYL, which may effectively close the N-terminal sequence H-S- that is highly sensitive to the dipeptidyl peptidase IV that widely exists in organism. And closing of enzyme-sensitive sequences at the N-terminus may improve the stability and half-life of the peptide in vivo. From bioactivity assay of DBAYL, the methionine at the N-terminus should have no effect on the high flexibility of the N-terminal region of DBAYL, and the receptor potency of DBAYL at human VPAC2 maintains highly selective activity. Except for the methionine at the N-terminus of DBAYL, other three mutations (N10Q, V18L, and N29Q) were simultaneously introduced into the peptide sequence by DNA recombination to avoid deamidation and improve the soluble (data not shown).
The recombinant DBAYL may be efficiently prepared through the strategy in this report. The prepared recombinant DBAYL has high biological activity. DBAYL may selectively bind to and activate VPAC2 receptor, and the receptor potency of DBAYL at human VPAC2 is higher than that of rPACAP38 or BAY55-9837. These results showed that the prepared recombinant DBAYL was a VPAC2-specific agonist with high potency and bioactivity.

Acting as a VPAC2-specific agonist, DBAYL can significantly induce insulin-stimulated IRS1 and GLUT4 expression in differentiated 3T3-L1 adipocytes. IRS1 is a key and essential protein for insulin signal transduction and GLUT4 is an important rate-limiting factor of the glucose transport. IRS-1 plays a major role in the activation of phosphatidylinositol 3-kinase, which is essential for GLUT4 translocation [29,30]. In human obesity and type 2 diabetes, the nositol 3-kinase, which is essential for GLUT4 translocation in differentiated 3T3-L1 adipocytes. IRS-1 plays a major role in the activation of phosphatidylinositol 3-kinase, which is essential for GLUT4 translocation [29,30]. In human obesity and type 2 diabetes, the receptor potency of DBAYL at human VPAC2 is higher than that of rPACAP38 or BAY55-9837. These results showed that the prepared recombinant DBAYL was a VPAC2-specific agonist with high potency and bioactivity.

Several reports showed that IRS1 and GLUT4 expression may provide the conditions for improving insulin signal transduction and glucose transport. Furthermore, the insulin-stimulated GLUT4 translocation to the plasma membrane was increased by 51% in differentiated 3T3-L1 adipocytes treated with 1 μM DBAYL, which was agreed well with the glucose uptake results. In glucose uptake activity assay, glucose uptake of differentiated 3T3-L1 adipocytes treated with 1 and 5 μM DBAYL was increased by 43% and 49%, respectively. Several reports showed Akt is probably involved in insulin-induced glucose uptake [33,34]. Dominant-negative Akt (mutations K179A, T308A, and S473A protein kinase B/Akt) markedly inhibits insulin-induced GLUT4 translocation, so Akt/PKB may play a key role in the process of insulin-induced GLUT4 translocation [35,36]. Our study showed that the recombinant DBAYL effectively promoted insulin-stimulated IRS1 and GLUT4 expression and GLUT4 translocation in a non-insulin-dependent manner. Corresponding to these results, glucose uptake activity of differentiated 3T3-L1 adipocytes-treated DBAYL were significantly improved, and the improvement effect of DBAYL was better than BAY55-9837.

In ICR mice, the recombinant DBAYL can obviously promote the insulin release and decrease the level of plasma glucose in a glucose-dependent manner. Increase of the insulin and other key proteins expression induced by DBAYL may be due to the fact that DBAYL potently stimulated cAMP generation, and as an important second messenger, cAMP increased the insulin and some proteins gene transcription and expression through cAMP/PKA signal pathway. Unlike rPACAP38, DBAYL can effectively decrease the plasma glucose level of ICR mice after glucose gavage through specifically activating VPAC2 receptor, and the biological effects of DBAYL are significantly better than BAY55-9837.

In summary, selective activation of VPAC2 by DBAYL can result in enhancement of glucose uptake, glucose-mediated insulin secretion and concomitant increase in glucose disposal without hypoglycemia. These results suggest that a novel VPAC2-selective agonist may serve as a therapeutic of type 2 diabetes.

### Funding

This work was supported by the grants from the Cooperation Project in Industry, Education and Research of Guangdong Province and Ministry of Education in China (No. 2010B090400544), Guangzhou Municipal Science and Technology Program (No. 2011J4300112), the Fundamental Research Funds for the Central Universities (No. 21612408), the Natural Science Foundation of Guangdong Province (No. S201201008756), the National ‘863’ Project (No. 2006AA02Z125), the National Technical Innovation Fund for Medium and Small-Size Enterprise, China (No. 11C26214413218), and Guangzhou Municipal Science and Technology Program (No. 2010Z2-C321).

### References

PACAP-derived peptide promotes glucose uptake and glucose-dependent insulin secretion


