Staurosporine as an agonist for induction of GLUT4 translocation, identified by a pH-sensitive fluorescent IRAP-mOrange2 probe

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ABSTRACT

Insulin-stimulated GLUT4 translocation from GLUT4 storage vesicles (GSVs) to the plasma membrane (PM) constitutes a key process for blood glucose control. Therefore, compounds that could promote GLUT4 translocation into the PM represent potential drugs for the treatment of diabetes. In this research, we screened for agonists that induce GLUT4 translocation by using a novel pH-sensitive fluorescent probe, insulin-regulated aminopeptidase (IRAP)-mOrange2. We identified as well as validated one agonist, staurosporine, from a 64,000 compound library. Staurosporine promotes GSVs translocation into the PM and increases glucose uptake through the AMP-activated protein kinase (AMPK) pathway, serving as an effective insulin additive analogue in L6 cells. Our work highlights the convenience and efficiency of this novel pH-sensitive fluorescent probe and reveals the new biological activity of staurosporine as an agonist for GLUT4 translocation and as an effective insulin additive analogue.

1. Introduction

As one of the major epidemic diseases, diabetes seriously threatens our health. According to statistics from the World Health Organization, there will be more than 300 million diabetic patients in 2025 [1]. It is now widely accepted that the pathological causes of diabetes lie in insulin resistance and/or impaired insulin secretion. Commercially available anti-diabetic therapies include insulin injection, as well as oral medications, such as sulphonylureas [2,3], biguanides [3], α-glucosidase inhibitors [3,4], and insulin sensitizers (TZD) [3,5]. Among these drugs, TZD improves insulin sensitivity and decreases insulin resistance, but it cannot be widely used in clinical treatment because of side effects such as weight gain, liver damage and increased cardiac stress [5]. Although biguanides are frequently used in clinical therapy, they cause severe gastrointestinal disorders and pose a rare but life-threating risk of lactic acidosis. Animal or human insulin benefits patients with impaired insulin secretion but cannot effectively address insulin resistance. In addition, animal insulin has strong antigenicity, often leading to adipocyte dissolution and necrosis after long-term injection. Even human insulin has side effects such as hypoglycemia [6–8]. Therefore, it is necessary to develop novel anti-diabetes drugs such as new insulin analogues and/or additives that could effectively stimulate glucose uptake and safely restore the diabetic patients' blood glucose to normal levels.

GLUT4, a glucose transporter expressed mainly in skeletal muscle cells and adipocytes, is crucial for maintaining blood glucose homeostasis in the human body. In the resting state, GLUT4 is mainly located in intracellular GSVs and cannot transport glucose. Upon stimulation by insulin, intracellular GSVs are translocated to and fused with the PM. The vesicle luminal domain of GLUT4 is then exposed to the extracellular matrix and ultimately transports glucose into cells [9]. One of the main problems of diabetic patients is that GLUT4 cannot be translocated into the PM and transport glucose into cells when blood glucose becomes higher

after a meal. Type I diabetes is due to insufficient insulin secretion by diminishing pancreatic beta cells, and type II diabetes mostly results from the inefficient response of skeletal muscle cells and adipocytes to insulin stimulation, including the lack of GLUT4 translocation into the PM [10]. Hence, small molecule compounds that promote GLUT4 translocation into the PM by mimicking insulin or overcoming insulin resistance might be developed for the treatment of diabetes.

In the present study, because both the N- and C-termini of GLUT4 face the cytosol, we attached a pH-sensitive mOrange2 fluorescent protein to the C terminal of IRAP, which co-localizes with GLUT4 and can be used to monitor GLUT4 translocation in real time in live cells simply by fluorescence measurement [11]. With the fusion of GSVs with the PM and GLUT4 translocation, the C-terminus tagged mOrange2 on IRAP will sense the pH level change from acidic vesicle lumen to extracellular neutral medium, and the fluorescence of mOrange2 will increase dramatically as a result (as shown in Fig. S1A). Based on this novel pH-sensitive fluorescent probe, IRAP-mOrange2, we carried out high-throughput screening, identifying and validating a hit compound, staurosporine.

Our work highlights the convenience and efficiency of this novel pH-sensitive fluorescent probe, including its ability to provide quantitative measurements in real time, and reveals the new biological activity of staurosporine as a GLUT4 translocation agonist and an effective insulin additive analogue.

2. Materials and methods

2.1. Materials and reagents

Fetal bovine serum, α-Minimum Essential Medium and penicillin/streptomycin were purchased from GIBCO. The 96-well optical glass bottom plates for drug screening were from Thermo Scientific Nunc. The screening compound library was provided by the National Drug Screening Center in Shanghai, China. Insulin, Compound C, wortmannin and β-actin antibody were from Sigma. AKT, AMPK/Thr172 and AKT/T308 antibodies were from Cell Signaling Technology.

2.2. Development and optimization of a cell-based, high-throughput screening assay with a novel pH-sensitive fluorescent probe IRAP-mOrange2

As previously reported, we have developed IRAP-pHluorin as a robust and reliable real-time probe for monitoring GLUT4 vesicle fusion in adipocytes [11]. To develop a cell-based, high-throughput screening assay, we adopted mOrange2 rather than pHluorin, in order to reduce the signal interference caused by green cellular auto-fluorescence and to increase the S/B (signal/background) ratio of the assay.

We established the stable IRAP-mOrange2-expressing cell line on L6 myoblasts which contain all the essential components from either the AKT or the AMPK signaling pathway for GLUT4 translocation. We didn't selected preadipocyte 3T3-L1 cells because the retrovirus infection procedure will render 3T3-L1 cells unhealthy. L6 myoblasts were infected with IRAP-mOrange2 packaged retroviruses. The positive cells were sorted by FACSAria Cell Sorter and seeded into 96-well plates. Monoclonal cells were picked and cultured. We selected the best-performing clone, named L6.IRAP-mOrange2. As demonstrated in Fig. S1B and S1C, we found that upon insulin stimulation, the membrane fluorescence of this clone under TIRFM increased gradually and reached a plateau after 30 min. The S/B ratio was 2.02, the coefficient of variation was 10.2, and the Z factor was 0.35 at 30 min, indicating that the assay

performance reached the criteria for high-throughput screening. Additionally, we measured the dose response curve for insulin in this clone; the calculated EC_{50} is 83.7 nM (Fig. S1D), lower than the published EC_{50} of insulin on L6 cells (149 nM) [12], indicating that our newly developed assay is more sensitive to insulin stimulation.

In order to maintain the stability and a large signal window during high-throughput screening, we evaluated the influence of some experimental conditions such as reagent source, experimental procedure and DMSO concentration on L6.IRAP-mOrange2 cells. Insulin from Sigma served as the positive control because of its sustained high activity during the repeated trials. Insulin was applied at 200 nM, and the image capture duration was set to 30 min, during which time insulin can promote maximal GSVs translocation into the PM and elicit the highest membrane fluorescence intensity. The signal window is sensitive to two other factors, cell culture time and DMSO concentration. In the screening assay, cells were cultured for 72 h, and the initial cell seeding density was 2.5×10^3 cells per well in 96-well plates. We chose a final DMSO concentration of 0.5%, which means that 0.2 mM compounds in DMSO were diluted 200-fold to a final working concentration of 1 μM.

Compared to the previously reported GLUT4 translocation assays [13–16], our IRAP-mOrange2 probe is more convenient and efficient. We could quantitatively measure the level of fluorescence enhancement and directly monitor the translocation of GLUT4 in real time, avoiding the time-consuming and expensive immuno-staining procedures.

2.3. Membrane fluorescence imaging

Cells were seeded into glass bottom dishes (1 mm). After serum-starvation for 2 h, the extracellular solution was replaced by KRBB (129 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 3 mM glucose, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.1% bovine serum albumin (pH 7.2)), and IRAP-mOrange2 would be translocated into the PM upon stimulation by 100 nM insulin. Images were captured by total internal reflection fluorescence microscopy (TIRFM).

2.4. Wide-field cell fluorescence imaging

The high-throughput fluorescence image acquisition system was composed of an Olympus IX81 microscope equipped with an Olympus 20* (NA = 0.75) air lens, fluorescent filter, Andor CCD camera, XY Stage, and TILL laser light source. Images were acquired using the Micro-manager program. Data were analyzed with Image J.

2.5. Glucose uptake

Cells in 12-well plates were washed three times with KRBH buffer (20 mM NaCl, 25 mM Hepes, 4.6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.9 mM $CaCl_2$, pH 7.4), unpretreated or pretreated with wortmannin (15 min pre-incubation) or Compound C (30 min pre-incubation), and stimulated by staurosporine for 2 h or by insulin for 15 min. After incubation, KRBH buffer with 3H-2DG (1 μCi/mL) and 2DG (50 μM) was added into each well, cells were incubated at 37 °C for 5 min, placed on ice immediately and washed 3 times with ice-cold KRBH buffer, and 300 μL of 1% SDS lysis buffer was added into each well. The cell lysate was transferred into scintillation vials with 3 mL of scintillation fluid and mixed thoroughly. Radioactive signals were detected by a liquid scintillation counter (Perkin-Elmer). Each experiment was repeated in 3 sets, the average and standard deviation was calculated, and the *t*-test was used to assess the significance of differences.