Original Article

Establishment of a cell-based assay to screen insulin-like hypoglycemic drugs

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ABSTRACT: This study sought to establish a cell-based assay to screen insulin analogs. Previous studies have proposed that up-regulation of glucose consumption may have the same anti-diabetic effects as insulin. Here, the amount of glucose that disappeared in culture medium after incubation with insulin or drugs was determined and served as an indicator of the glucose consumption of the cells. In order to establish a cellular model to screen insulin analogs, the sensitivities of four cell lines - BALB/c 3T3, HepG2, NIH3T3, and Bel7402 - to insulin were evaluated by detecting glucose consumption after incubation with insulin (0-125 nM) for 24 h. BALB/c 3T3 was more sensitive to insulin than the other three cell lines. Insulin elevated glucose consumption of BALB/c 3T3 in a concentration- and time- manner. Glucose consumption of BALB/c 3T3 increased by 30% after incubation with insulin (30 nM) for 24 h. Insulin increased the proliferation of BALB/c 3T3 at 48 h. A model was established by detecting glucose consumption after treating BALB/c 3T3 with drugs for 24 h. Using the cell-based assay, we screened more than two thousand samples from Traditional Chinese Medicine (TCM). Five extracts exhibiting glucose absorbance in medium were identified, indicating a hit rate of 0.5%. Results suggested that a cell-based assay by detection of glucose consumption in BALB/c 3T3 was suitable for high-throughput screening and was feasible to identify insulin-like hypoglycemic drugs. Five hits were discovered from natural products. Further characterization of these active extracts could help to identify potential anti-diabetic drugs.

Keywords: BALB/c 3T3 cell, Insulin analogs, Glucose consumption, Drug screening

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1. Introduction

Diabetes is a chronic metabolic disorder affecting approximately five percent of the population of industrialized nations. Insulin is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism. The pathogenesis of type II diabetes includes insulin resistance and a relative deficiency in insulin secretion. Insulin stimulates a variety of cellular metabolic changes, such as glucose uptake and glycogen and lipid synthesis. The main assays for activity of insulin analogs have been reported to use radioisotopes (*1,2*). In order to find leading compounds for new insulin-like hypoglycemic drugs, a simple and sensitive high-throughput screening model that does not require radioisotopes must be established.

This study established a cell-based screening method by assaying glucose consumption. More than 2,000 samples including compounds and natural products were screened using this cellular model. The samples were derived from Traditional Chinese Medicine (TCM). DF007, DF052, DF167, DF262, and DF432 (lab serial numbers) were identified as accelerating glucose consumption in a BALB/c 3T3 cell line. These active extracts may provide potential anti-diabetic drugs and warrant further study.

2. Materials and Methods

2.1. *Reagents*

Insulin, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide), Pluronic F68, and a Glucose detection kit were obtained from Sigma. Dulbecco's modified Eagle's minimum essential medium (DMEM), RPMI 1640 medium, and Fetal bovine serum (FBS) were purchased from GIBICO. Metformin hydrochloride was purchased from Beijing Liling pharmaceutical Co. and dissolved in distilled normal saline.

2.2. *Cell lines*

BALB/c 3T3 was purchased from the Cell Center of Wuhan University. HepG2, NIH3T3, and Bel7402 were purchased from the Cell Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

2.3. *Maintenance of cells*

BALB/c 3T3 and HepG2 were maintained in DMEM culture medium including 10% FBS, 2 mM glutamine, 100 kU/L penicillin, 100 mg/L streptomycin, and a high glucose concentration (4.5 g/L) at 37°C, 5% CO_2. NIH3T3 and Bel7402 were maintained in culture medium RPMI1640 including 10% FBS, 2 mM glutamine, 100 kU/L penicillin, and 100 mg/L streptomycin at 37°C, 5% CO_2. Cells cultures that became confluent in culture flasks were used in the glucose consumption assay.

2.4. *Glucose consumption assay*

Cells were detached from the culture flask with a solution of 0.25% trypsin and 1 mM EDTA. Trypsin digestion was stopped by the complete culture medium. The cells were washed twice and resuspended in low-glucose (1.0 mg/mL) detection medium supplemented with 0.05% Pluronic F68, 0.2% bactopeptone, and 2 mM glutamine. The cell density was regulated to a concentration of 1×10^5/mL and cells were spread onto 96-well microtiter plates (100 μL per well). The cells were cultured with serial insulin (final concentration 0 - 125 nM) or samples at 37°C, 5% CO_2 for 4-48 h. At the end of incubation, 10 μL suspension per well or glucose standard medium (0-1,000 mg/L) was moved to another 96-well plate well by well. The glucose concentration remaining in suspension was measured by illumination in a glucose assay. Briefly, the reaction lasted 30 min at room temperature. The absorbance at 495 nm was determined with a Polarstar Microplate Reader. The glucose concentration left in medium was calculated by the standard curve of glucose. The percent of glucose consumption (*R*gc) was calculated using the following formula and IC_{50} was determined graphically.

$$Rgc = (A–B)/(A–C) \times 100$$

A, concentration of glucose in medium for the control; B, concentration of glucose in medium for sample groups; C, a blank control without cells in the same medium with the control. The glucose concentration was given in μg/mL.

2.5. *MTT assay*

BALB/c 3T3 cells were seeded at a density of 1×10^4 cells per well in 96-well microtiter plates and cultured with insulin (30 nM) in detection medium for 24-48 h at 37°C in an atmosphere 5% CO_2. The cells were stained with MTT by a modification of the method of Mosmann (*3*). Briefly, the suspension medium was removed and 100 μL MTT (final concentration 5 mg/L) were added to each well 4 h before the end of incubation. After culturing for 4 h, the suspension was discarded and 150 μL dimethyl sulfoxide (DMSO) per well were added. Absorbance was read at 540 nm with a Polarstar microplate reader.

2.6. *Natural product extracts*

All samples were from the Sample Library of this institute. Each TCM (2 kg) was finely milled and extracted with petroleum ether, 95% alcohol, and water in turn. The samples were dissolved in DMSO and diluted with normal saline. The final DMSO concentration was less than 0.1%.

2.7. *Statistical analysis*

Data were given as the mean ± S.D. and the differences were calculated with a Student's two-tail *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. *Sensitivity of the four cell lines to insulin*

BALB/c 3T3, HepG2, NIH3T3, and Bel7402 cell lines were incubated with serial diluted insulin for 24 h. Glucose consumption in medium was compared in different cells stimulated by insulin. As shown in Figure 1, insulin lowered glucose concentration in suspensions of BALB/c 3T3 and HepG2 in a dose-dependent manner. Glucose concentration tended to decrease in the suspension of Bel7402, but there was no change in glucose uptake of NIH3T3 in comparison to the control. BALB/c 3T3, HepG2, and Bel7402 were found to be sensitive to insulin. Of the cell lines, BALB/c 3T3 in particular had a high level of sensitivity, indicating its potential for use in a cell assay to screen hypoglycemic drugs.

3.2. *Effect of insulin on glucose consumption in the BALB/c 3T3 cell line*

Highly sensitive to insulin, the BALB/c 3T3 cell line was used to observe the dose-response and linear response curve of insulin on glucose consumption. As shown in Figure 2, insulin-accelerated glucose consumption in BALB/c 3T3 in a concentration- and time-dependent manner. Insulin of 31.25 nM was able to accelerate glucose consumption by 30% (Figure

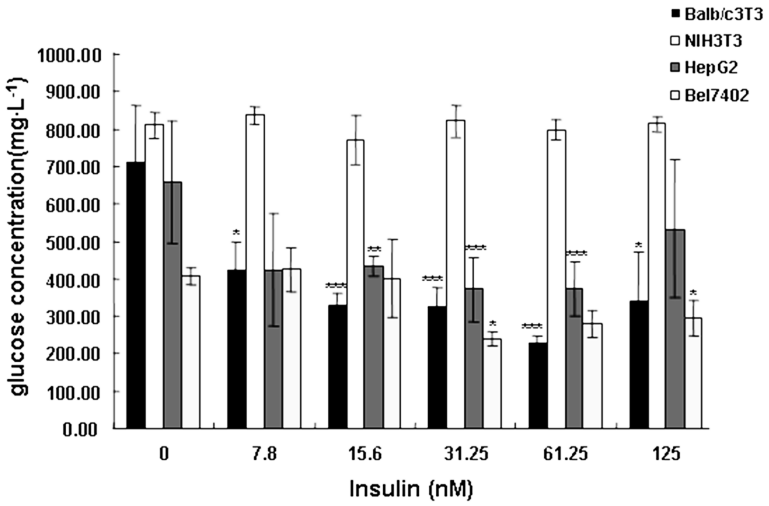

Figure 1. Sensitivity of different cells to insulin. BALB/c 3T3, NIH3T3, HepG2, and Bel7402 were treated with or without different concentrations of insulin for 24 h. Then, the glucose concentration remaining in suspension was measured as described in the Methods. All data are expressed as mean ± S.D. (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* control cells without insulin treatment.

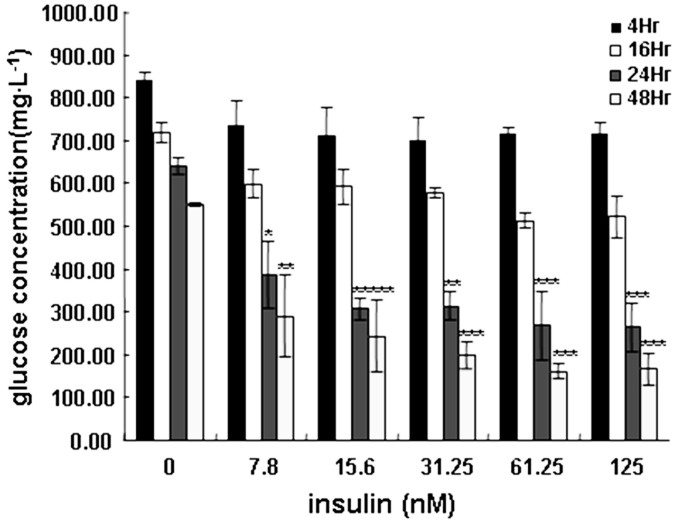

Figure 2. Dose-effect and time-dose relationships for insulin-induced glucose consumption in BALB/c 3T3. BALB/c 3T3 cells were treated with or without different concentrations of insulin for 24 h. The glucose concentration remaining in suspension was measured as described in the Methods. Values are expressed as mean ± S.D. (n = 8). **P < 0.01 compared to control cells without insulin treatment.

2). Glucose consumption at 24 h or 48 h was higher than that at 4 h and 16 h. In order to test the glucose consumption of BALB/c 3T3, the cells should be cultured with insulin for at least 24 h.

3.3. *Effect of insulin on cell proliferation of the BALB/c 3T3 cell line*

Because the glucose consumption was related to the number of cells, an experiment was performed to test cell proliferation. As shown in Table 1, insulin increased the proliferation of BALB/c 3T3 in time-dependent manner. There was marked difference between 31.25 nM insulin and the control at 48 h, and there was no significant difference at 24 h. An incubation time of 24 h proved suitable at eliminating the influence of cell numbers on glucose consumption.

Table 1. Effect of insulin on proliferation of BALB/c 3T3. BALB/c 3T3 cells were treated with or without insulin for 24 h or 48 h. Then, cell viability was determined by MTT assay. All data are presented as the mean ± S.D. of at least three independent experiments, n = 8

Insulin (nM)	24 h (OD)	48 h (OD)
0	0.89 ± 0.06	0.94 ± 0.05
7.8	0.87 ± 0.03	1.04 ± 0.08
15.6	0.92 ± 0.05	1.19 ± 0.04
31.25	0.94 ± 0.06	1.29 ± 0.05[a]
62.5	0.96 ± 0.09	1.29 ± 0.09[a]
125	0.99 ± 0.08	1.35 ± 0.06[a]

[a] P < 0.05 compared to the control.

3.4. *Effect of metformin hydrochloride on glucose consumption in the BALB/c 3T3 cell line*

Metformin hydrochloride accelerated glucose consumption in the BALB/c 3T3 cell line in a

Table 2. Effect of metformin hydrochloride on glucose consumption in BALB/c 3T3. BALB/c 3T3 cells were treated with or without insulin or metformin for 24 h. The glucose concentration remaining in suspension was measured as described in the Methods. Values are expressed as mean ± S.D. (n = 8)

Group	Dose (M)	Glucose (mg/L)	%[a]
Control	-	644.56 ± 55.79	
Insulin	3×10^{-8}	423.95 ± 31.04[b]	34.99
Metformin hydrochloride	7.55×10^{-4}	443.43 ± 18.5[b]	31.90
Metformin hydrochloride	7.55×10^{-5}	479.07 ± 22.63[b]	26.24
Metformin hydrochloride	7.55×10^{-6}	484.33 ± 20.95[b]	25.41
Metformin hydrochloride	7.55×10^{-7}	536.34 ± 47.33	17.16
Metformin hydrochloride	7.55×10^{-8}	753.14 ± 32.67	17.22

[a] Glucose uptake (% of control) = (A–B)/(A–C) × 100. A, control; B, sample; C, blank.
[b] $P < 0.01$ compared to the control.

Table 3. Effect of test samples on glucose consumption in BALB/c 3T3 (n = 8)

Serial number of sample	Materia	Extract	IC_{50} (mg/L)
Control			-
DF007	*Alpinia oxyphylla Miq.*	Ether	84.48
DF052	*Croton tiglium L.*	Ether	53.72
DF167	*Angelica sinensis Diels.*	Ethanol	36.60
DF262	*Fructus trichosanthis*	Ethanol	58.50
DF432	*Polygonatum cyrtonema*	Ethanol	13.12

concentration-dependent manner. At a concentration of 7.55×10^{-6} M, metformin hydrochloride accelerated consumption at a rate of about 25.41% (Table 2).

3.5. *Effect of test samples on glucose consumption in the BALB/c 3T3 cell line*

There were more than 2,000 samples for screening using the model. DF007, DF052, DF167, DF262, and DF432 were identified as significantly accelerating glucose consumption and warranting further investigation. These samples exhibited an IC_{50} at 84.48, 53.72, 36.60, 58.50, and 13.12 mg/L (Table 3). These samples were extracted from TCM. DF007 and DF052 were petroleum ether extracts from *Alpinia Oxyphylla* and *Croton Tiglium*, respectively. DF167, DF262, and DF432 were EtOH extracts from *Angelica Sinensis Diels*, *Fructus Trichosanthis*, and *Polygonatum*, respectively.

4. Discussion

Type II diabetes accounts for the vast majority of cases of diabetes (*4*). Discovery of oral insulin-mimetic agents has been a long-standing goal of pharmaceutical research. The target cells of insulin include fat cells, hepatic cells, muscle cells, and fibrosis cells. Several reports showed that 3T3-L1 (a prefat cell) provided a cell model to study the mechanism of insulin resistance or effects of anti-hyperglycemic agents (*5,6*). In the current study, the four cell lines of BALB/c 3T3 (a fibrosis cell line), HepG2 (a hepatic cell line), Bel7402 (a hepatic cell line), and NIH3T3 (a fibrosis cell line) were used to develop a high-throughput screening model by metabolic assay. BALB/c 3T3 is a fibroblast cell line that has been used to quantify insulin-like growth factor (IGF) bioactivity with stimulation of cell proliferation and glucose consumption (*7*). HepG2 is a hepatic embryo tumor cell line and has the same morphology and function of hepatic cells (*8*). Bel7402 was from a human hepatic tumor. The current study found that NIH3T3 was not sensitive to insulin, and glucose concentration in remaining medium tended to decrease for HepG2 and Bel7402. NIH3T3 was not sensitive to insulin because it had no endogenous insulin receptors. However, the results did indicate that BALB/c 3T3 had a high level of sensitivity to insulin, making it the best candidate cell line for use in the screening model. MTT assay revealed that insulin (31.25 nM) did not cause a change in the number of BALB/c 3T3 cells after 24 h of culturing. Results demonstrated that the effect of cell proliferation on glucose consumption was minimized by incubating BALB/c 3T3 cells with insulin or samples for 24 h.

Results showed that in BALB/c 3T3 cells metformin hydrochloride reduced levels of glucose remaining in medium. This suggests that the cell-based assay is appropriate and consistent. TCMs have historically been a prolific source of therapeutically useful drugs. Results also identified five active samples that were petroleum ether extracts or EtOH extracts from TCMs; the glucose consumption of these samples was evaluated using high-throughput screening. In order to identify leading compounds, these samples must be further separated and purified and subjected to structure-activity analysis and structural modification.

Of course, active samples identified by this model may have many potential effects, such as activating insulin and insulin growth factor (IGF) receptors, stimulating Glut4 transporter, or activating tyrosine kinase (IRTK) activity. In order to identify the mechanism at work, positive samples should be tested for activation of insulin receptor signaling. Phosphorylation of insulin receptor substrate-1 could be tested by using SDS-PAGE and Western blotting analysis. If they activate IRS-1 phosphorylation, the samples may be insulin-mimetic agents.

The indicator used by the cell-based assay was glucose consumption. Traditional methods of detecting glucose metabolism included measuring glucose absorbance or detecting hepatic products and fatty acids. These assays require radiolabeled metabolites. In the current study, however, glucose consumption was assayed colorimetrically with no need for radioisotopes. In conclusion, this cell-based assay is simple, inexpensive, and suitable for screening of insulin-like hypoglycemic drugs.

Acknowledgments

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