Acebutolol, a Cardioselective Beta Blocker, Promotes Glucose Uptake in Diabetic Model Cells by Inhibiting JNK-JIP1 Interaction

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Abstract

The phosphorylation of JNK is known to induce insulin resistance in insulin target tissues. The inhibition of JNK-JIP1 interaction, which interferes JNK phosphorylation, becomes a potential target for drug development of type 2 diabetes. To discover the inhibitors of JNK-JIP1 interaction, we screened out 30 candidates from 4320 compound library with In Cell Interaction Trap method. The candidates were further confirmed and narrowed down to five compounds using the FRET method in a model cell. Among those five compounds, Acebutolol showed notable inhibition of JNK phosphorylation and elevation of glucose uptake in diabetic models of adipocyte and liver cell. Structural computation showed that the binding affinity of Acebutolol on the JNK-JIP1 interaction site was comparable to the known inhibitor, BI-78D3. Our results suggest that Acebutolol, an FDA-approved beta blocker for hypertension therapy, could have a new repurposed effect on type 2 diabetes elevating glucose uptake process by inhibiting JNK-JIP1 interaction.

Key Words: Acebutolol, JNK inhibitor, Glucose uptake, Drug screening, Diabetes Mellitus

INTRODUCTION

The role of c-Jun N-terminal kinase (JNK) in diabetes has been studied over the past decade. Activated JNK contributes to the inactivation of TNFα-induced insulin receptor subunit 1 (IRS1) by Ser307 phosphorylation, leading to insulin resistance (Aguirre et al., 2000). JNK is also involved in the suppression of transcription of insulin, Glut2 and PPARγ (Kaneto et al., 2002). Moreover, JNK phosphorylation in adipose tissue was elevated in type 2 diabetes patients compared to healthy people (Carlson et al., 2003). Because of the central role of JNK in insulin resistance and pancreatic β-cell dysfunction, inactivation of JNK becomes a new therapeutic target for antidiabetic drug development (Hirosumi et al., 2002; Bennett et al., 2003).

There have been considerable efforts to develop JNK inhibitors, initially focused on ATP-competitive inhibitors like SP600125 and its derivatives, then expanded to the other small compound groups, natural product sources, and peptide inhibitors (Bogoyevitch and Arthur, 2008). Peptide inhibitors were designed to interfere the interaction of JNK with c-Jun, its downstream substrate, or JIP1, a cytoplasmic scaffold protein that interacts with JNK and activates JNK phosphorylation (Bogoyevitch and Arthur, 2008). Even though several roles of JNK in diabetes mechanisms, only a few JNK inhibitors have been verified for antidiabetic activities. Several competitive peptide inhibitors were developed for JNK inhibitors targeting the JNK interacting site of JIP1 (Barr et al., 2002; Heo et al., 2004). Stebbins and colleagues further developed an improved compound, BI-78D3, which mimics a JNK interacting peptide, pepJIP1 (Stebbins et al., 2008). BI-78D3 could inhibit JNK phosphorylation by disrupting JNK-JIP1 interaction and successfully restrained insulin resistance in the mouse models of type 2 diabetes like pepJIP1. In addition, BI-78D3 enhance the in vivo efficacy that was limited with the peptide-based inhibitor like pepJIP1. These studies showed clearly the potential of JNK-JIP interaction site as a target of antidiabetic drug development but the number of verified inhibitors are very limited yet.

To expand antidiabetic drug candidates targeting JNK-JIP1 interaction, we screened 4320 library compounds including 1280 pharmacologically active compounds and 1920 approved drugs with In Cell Interaction Trap (InCell IT) method (Kim et al., 2008) and fluorescence resonance energy transfer (FRET) assay. We selected five compounds having significant inhibition of JNK-JIP1 interaction. Among those selected
candidates, Acebutolol showed significant antidiabetic characteristics that suppress JNK phosphorylation and promotes glucose uptake in model cells, which is comparable to the known inhibitor, BI-78D3. Computational simulation revealed that the binding affinity of Acebutolol on JNK-JIP1 interaction site is comparable to that of BI-78D3. These findings provide the evidences that Acebutolol has antidiabetic effects elevating glucose uptake process by inhibiting JNK-JIP1 interaction in model cells. We suggest the further in vivo studies to achieve repurposed therapeutic application of Acebutolol on type 2 diabetes in addition to its original therapeutic function on hypertension.

MATERIALS AND METHODS

Materials

Total 4320 screening compound libraries were purchased from Sigma-Aldrich (St. Louis, MO, USA) (1280 compounds of LOPAC), Enzo Life Sciences (Seoul, Korea) (640 compounds of FDA approved drug library), Tocris Bioscience (Bristol, UK) (1120 compounds of Tocriscreen compound library collection) and Microsource Discovery Systems (Gaylordsville, CT, USA) (1280 compounds of US drug collection). Full-length of JNK cDNA (GenBank Accession No. NM_002750.2) and JIP1 cDNA (GenBank Accession No. BC068470) were obtained from Open Bio (Seoul, Korea) and Imagenes (Seoul, Korea) respectively. BI-78D3 was purchased from EMD Milipore (Billerica, MA, USA). TNFα was purchased from R&D Systems (Minneapolis, MN, USA) and insulin was purchased from Roche (Seoul, Korea). Lipofectamine 2000, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) and 293A cell were purchased from ThermoFisher Scientific (Waltham, MA, USA). HeLa, HepG2 and 3T3L1 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Anti-phospho-JNK was purchased from Abcam (Cambridge, MA, USA), anti-JNK was purchased from Santa Cruz (Dallas, TX, USA) and anti-actin was purchased from Sigma-Aldrich. Secondary mouse and rabbit antibody were purchased from The Jackson Lab (Farmington, CT, USA).

Plasmid constructions

Full-length of JNK and JIP1 cDNA were cloned into pEGFP-N1 and pmRFP-C3 mammalian expression vectors by conventional molecular cloning for the InCell IT screening respectively. For FRET screening, JNK and JIP1 full-length cDNA were subcloned into pREST-CFP and pEYFP-N1 mammalian expression vectors respectively. These cloned products were verified through DNA sequencing.

Cell culture and transfection

HeLa and HepG2 cells were maintained in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% Fetal Bovine Serum (FBS). 293A Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS. Pre-differentiation of 3T3L1 cells were maintained in DMEM with 10% Bovine Serum (BS). Differentiation of 3T3L1 cells were induced by DMEM containing 10% FBS with 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM dexamethasone, and 10 μg/ml insulin and were grown for 3 days. And then, the media was changed to DMEM containing 10% FBS with 10 μg/ml insulin for two more days. All cells were grown in 5% CO2 at 37°C in a humidified environment. Cells were transiently transfected using Lipofectamine 2000 according to the manufacturer’s instructions. For FRET screening, 293A cells were cultured in 96-well plate for 24 h. Then, JNK-EGFP and JIP1-mRFP with linked magnetic nanoparticles were co-transfected in this cell. After 24 h incubation, each compounds were incubated for one more hour respectively. And then these proteins are localized in cytosol with specific pattern depending on magnet field in the cell. Cells were then fixed with 4% paraformaldehyde. Fluorescence images were taken by fluorescence microscopy (Eclipse TS100, Nikon). For collection of positive candidates, compounds were selected by different fluorescence position of JIP1-mRFP and JNK-EGFP expression each in cell.

Screening of JNK-JIP1 interaction inhibitors

InCell IT screening was performed based on a modified version of the method published previously (Kim et al., 2008). Briefly, HeLa cells were cultured in 96-well plate for 24 h. Then, JNK-EGFP and JIP1-mRFP with linked magnetic nanoparticles were co-transfected in this cell. After 24 h incubation, each compounds were incubated for one more hour respectively. And then these proteins are localized in cytosol with specific pattern depending on magnet field in the cell. Cells were then fixed with 4% paraformaldehyde. Fluorescence images were taken by fluorescence microscopy (Eclipse TS100, Nikon). For collection of positive candidates, compounds were selected by different fluorescence position of JIP1-mRFP and JNK-EGFP expression each in cell.

JNK phosphorylation analysis

Whole cell lysate proteins were made by sonication with lysis buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 10% Glycerol, 1 mM Na3VO4, 50 mM NaF, 1 mM Phenylmethylsulfonyl fluoride (PMSF) and 0.05% SDS) containing a protease-inhibitor cocktail and incubated on ice for 30 min. For the western blot analysis, the supernatants were separated by SDS-PAGE using 10% gels and blotted transferred onto a polyvinylidenedifluoride (PVDF) membranes. The blots were then probed with primary antibodies (1:1000) for 1 h. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000) for 45 min followed by additional washing. Signal was detected by chemiluminescence (ECL, GE healthcare, Little Chalfont, UK) and recorded by imaging analyzer (ImageQuant LAS 4000 mini, GE healthcare).

2-Deoxyxyglucose uptake assay

2-Deoxyxyglucose uptake was measured using fluorescent 2-NBDG reagent according to the manufacturer’s instructions. Briefly, cells were cultured without serum for 16 h and then treated TNFα (20 ng/ml) for 6 h. After that, these cells were stimulated by positive compounds (1 μM) for 1 h respectively. Before measure of Deoxyglucose level, media was changed to phenol red-free media with 80 μM 2-NBDG for 10 min. The fluorescence was measured by using a fluorescence micro-reader at excitation of 485 nm and emission of 535 nm.

Docking simulation

A docking simulation between JNK and Acebutolol was performed in Discovery Studio (version 3.1, BIOVIA, San Diego, Ca, USA). A docking simulation between JNK and Acebutolol was performed in Discovery Studio (version 3.1, BIOVIA, San Diego, Ca, USA). A docking simulation between JNK and Acebutolol was performed in Discovery Studio (version 3.1, BIOVIA, San Diego, Ca, USA).
CA, USA). The template structure of JNK (PDB ID: 4IZY) was downloaded from Protein Data Bank (Berman et al., 2000) and a binding site sphere of 10 Å diameter was created from a midpoint of Arg127 and Cys163 that are known to be docked on JIP1 peptide. The 2D structures of Acebutolol, BI-78D3 and Cyproterone Acetate were collected from PubChem (Kim et al., 2016) and converted to 3D conformations with enumerating isomers and tautomers using “prepare ligands” option. After that, LibDock was performed with following options: 100 hotspots, 0.25 of docking tolerance, fast conformation generation and CHARm forcefield. While the simulation, docking poses were generated by matching the clustered apolar and polar hot spots of JNK and each ligand, and refined hydrogen bondings and steric potentials based on BFGS optimization algorithm. After that, LibDock score was calculated using piecewise linear potential (PLP) like function with scores between interacting atoms that considered hydrogen binding potential or a steric potential of 4 different atom types (Rao et al., 2007). All possible docking poses were generated based on polar and apolar interaction sites in the defined active sphere ranked by LibDock scores. We selected the most potent poses of each compounds.

**RESULTS**

**Screening of JNK-JIP1 interaction inhibitors**

To find inhibitors of JNK-JIP1 interaction, we screened 4320 compounds. One of the non-effective compounds used as a negative control and BI-78D3 (BI) used as the positive control. FRET fluorescence (lower panel) shows intensity of JNK-JIP1 interaction.

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**Fig. 1.** Screening of JNK-JIP1 interaction inhibitors. (A) Illustration of FRET fluorescence analysis with JNK-CFP and JIP1-YFP. (B) FRET assay was performed as indicated in methods. 0.001% Dimethyl sulfoxide (DMSO) is a vehicle control and Cyproterone acetate (CPA) was used as negative control. BI-78D3 (BI), a known inhibitory compound of JNK-JIP1 complex, was used as positive control. Compound candidates were named by abbreviations, Acebutolol (AC), Lithium chloride (LICL), Vincristine sulfate (VCR), Niacinamide (NAA), Valproic acid (VPA), Dihydro ouabain (DHO), Ethylisopropylamiloride (EIPA), Chloroquine diphosphate (CDP), Homosalate (HMS), Eniconazole (ENIL), Sertraline (SRT), 5-aminovaleric acid (5AVA), Methocarbamol (MCBL), Quinapril (QUI), Osetlomivir (OTV), Nitrofurantoin (NTF), Homatropine bromide (HB), Propranolol (PRO), JLK6, Indirubin-3'-monoxime (I3MO), Hydroquinone (HQ), O(6)-benzylguanine (O6BG), N-bromosuccinamide (NBA), PK 11195, Nifedipine (NIF), Probenecid (PROB), Yohimbine hydrochloride (YOH), Zacopride (ZAC), Ranitidine hydrochloride (RANH). The bar graph shows relative FRET intensity of the mean ratio of the FRET versus CFP from three independent experiments. Statistical analysis was assessed by one-tailed unpaired t-test; n=3; *p<0.05, **p<0.01, ***p<0.001 vs. DMSO. (C) JNK-CFP (upper panel) and JIP1-YFP plasmids were co-transfected in 293A cells, and then candidate compounds (1 μM) were treated for 1 h. DMSO represents the vehicle control with 0.001% DMSO. Cyproterone acetate (CPA) is one of the non-effective compounds used as a negative control and BI-78D3 (BI) used as the positive control. FRET fluorescence (lower panel) shows intensity of JNK-JIP1 interaction.
compounds in HeLa cells using InCell IT method for high-throughput primary screening and 30 positive candidates were selected as a result. Because there are several limitations of InCell IT system as difficult of protein interactive rate and high false positive ratio, we re-confirmed inhibition efficiency of 30 positive candidates by FRET analysis in 293A cells (Fig. 1A). Cells were transfected with JNK-CFP (donor) and JIP1-YFP (acceptor) and then stimulated with 458 nm wavelength. After FRET process, 535 nm emission wavelength were monitored by confocal microscopy. 22 out of 30 candidates decreased fluorescence signal of FRET significantly in JNK-CFP and JIP1-YFP co-transfected cells (Fig. 1B). BI-78D3 (BI), a known inhibitory compound of JNK-JIP1 complex, was used as positive control. Whereas Cypoterone acetate (CPA), one of non-effective drugs in InCell IT assay, was used as negative control (Fig. 1B). Although some limitation of InCell IT assay, this assay may be effectively high-throughput method for drug screening about protein-protein interaction.

Depending on inhibitory efficiency from FRET assay, we narrow down the candidates to top five positive inhibitors for further study. Selected candidates were Acebutolol, Lithium chloride, Vincristine sulfate, Niacinamide and Valproic acid. FRET images of final selected candidates together with controls were shown in the Fig. 1C.

Acebutolol prevents JNK phosphorylation

Although five candidates showed more than 40% inhibitory effect on JNK-JIP1 interaction from FRET assay, their contributions to JNK inhibition is still unknown. To identify direct relationship of candidates to JNK inhibition, phosphorylation level of JNK was tested in TNFα-treated 3T3L1 adipocyte and HepG2 liver cells, diabetic mimic cells.

To compare the effect of all drug candidates fairly and keep the consistence of all experiment condition, we continued to use the single does condition (1 μM) for second round selection. The does condition for drug candidates is decided as the same as optimized condition for control drug BI-78D3 to provide a guide of candidate selection. Treatment of TNFα increased phosphorylation of JNK in these cells (Fig. 2). Acebutolol and Lithium chloride decreased TNFα-induced JNK phosphorylation and Valproic acid and Niacinamide reduced JNK phosphorylation but small inhibition was achieved compared to Acebutolol or Lithium chloride in both cells (Fig. 2). However, phosphorylation of JNK was not inhibited but increased slightly by Vincristine sulfate in these cells (Fig. 2). Along with this result, Vincristine sulfate was also known to promote JNK phosphorylation in chronic lymphocytic leukaemia (CLL) cells (Bates et al., 2015). BI-78D3 was treated in parallel serving as positive control to confirm inhibitory efficiency of candidates. Interestingly, Acebutolol and Lithium chloride induced similar inhibitory effect of BI-78D3 in JNK phosphorylation of these cells (Fig. 2).

Based on the effect of both cells, we concluded that Acebutolol and Lithium chloride is more effective in inhibit of JNK activity among the candidates.

Glucose uptake increased by Acebutolol in insulin target cell lines

Because Acebutolol contributes to improve hypoglycemia in diabetic patients as well as it is an FDA-approved drug with clinical safety guarantee, Acebutolol was selected between effective candidates for further study (Deacon et al., 1977).
78D3 on glucose uptake is higher in normal cells than TNFα treated cells. Interestingly, in both cell conditions, ability of Acebutolol on glucose uptake is consistent (Fig. 3A). This can be interpreted as the effect of Acebutolol on glucose uptake is more specific to the model condition of insulin resistance: researchers use TNFα treatment for this model condition as TNFα inhibits insulin-stimulated glucose uptake.

Acebutolol and BI-78D3 enhanced approximately 50% glucose uptake but insulin increased approximately 20% glucose uptake compared with TNFα only treated 3T3L1 cells (Fig. 3B). With similar result of TNFα-treated 3T3L1 cells, glucose uptake was also enhanced in Acebutolol or BI-78D3 treated HepG2 cells compared with TNFα only treated HepG2 cells (Fig. 3C). These results show that Acebutolol increases glucose uptake effectively in these cells.

To figure out the relationship between Acebutolol effect and concentration, we performed dose-dependent study. In TNFα-induced diabetic model cells, Acebutolol showed dose-dependent response on glucose uptake that it significantly increases the glucose uptake as its concentration increased from 0.5 μM, 1.0 μM to 5.0 μM (Fig. 3D). On the other hand, in the absence of TNFα treatment, the glucose uptake at low concentration (0.5 μM) of Acebutolol reached to the similar level of high concentration (5.0 μM) of Acebutolol with TNFα-induced cells, and the effects were increased slightly at higher doses. This results explains well that Acebutolol can recover the glucose uptake from the suppressed level of TNFα-induced diabetic model cells to the level of TNFα-untreated normal condition.

Together with inhibitory effect of JNK phosphorylation, Acebutolol is a good candidate of JNK regulation in diabetic model cells.
Acebutolol binds to JNK protein of JIP1 binding site

To compare the binding regions and affinities between JNK and Acebutolol with BI-78D3, we performed the docking simulation by using LibDock analysis. The structure of Acebutolol and BI-78D3 showed in Fig. 4A and 4C respectively. The docking pose between Acebutolol and JNK shows that Acebutolol formed H-bonds with both Arg127 and Cys163 (Fig. 4B), same as that are shown in BI-78D3 (Fig. 4D). The average LibDock score of top 10 binding poses also implies that Acebutolol (65.1) has similar binding affinities with BI-78D3 (67.7) and these scores are ~2.9 higher than Cyproterone acetate (22.6) (Fig. 4E).

These results indicated that Acebutolol inhibits phosphorylation of JNK by the similar binding regions and affinities of BI-78D3 that acting as competitive binding component of JIP1.

DISCUSSION

In the present study, we identified in vitro antidiabetic effect of Acebutolol promoting cellular glucose uptake by interfering the JNK-JIP1 interaction and JNK phosphorylation consequently. Acebutolol reduced JNK-JIP1 binding (Fig. 1) and decreased phospho-JNK level in TNFα treated adipocyte (3T3L1) and liver cells (HepG2) (Fig. 2). Moreover, Acebutolol increased glucose uptake in these cells (Fig. 3). Furthermore, the LibDock score implies that the binding affinity of Acebutolol (74.9) on the JNK-JIP1 interaction site is similar to BI-78D3 (77.3). These results suggest that Acebutolol should be a comparable candidate with validated JNK-JIP1 inhibitors, BI-78D3 which could recover hyperglycemia symptom in mouse in vivo model. Based on our results, the further in vivo studies of Acebutolol is highly suggest to accelerate the introduction of diverse clinical choices on hyperglycemia therapy with this multi-functional drug.

Acebutolol (trade name Sectral) is a cardio-selective beta-adrenergic blocker currently in use for hypertension and cardiac arrhythmias without serious side effects since it was first introduced in the clinics more than 30 years ago (Charoenlarp and Jaronvesama, 1978). Interestingly, in the early stage of its clinical use, there was an issue of possible adverse effect of Acebutolol on glucose and lipid metabolism due to the adrenergic receptor signaling (Kumar et al., 2015). Some studies showed the possible hypoglycemic effect of Acebutolol on patients suffering from diabetes or both diabetes and hypertension (Fraser et al., 1986). Our present study provides more credibility on those unique clinical findings by revealing a possible cellular and molecular mechanism of the hypoglycemic effect of Acebutolol. It would be worth investigating the extended applicability of this drug on more diseases complicated with diabetes, or direct repurposing to type 2 diabetes.

Acebutolol could expand the flexibility of antidiabetic treatment by increasing the target diversity since there are only a few in vivo tested inhibitors and still no drug in use targeting JNK-JIP1 interacting site. Furthermore, considering diverse functions of JNK in cellular mechanisms and several JNK inhibitors developed for cancer and inflammation already (Kumar et al., 2015), there could be high potential to find additional effects of Acebutolol. We hope that our results could activate the investigation of diverse effects of Acebutolol as a JNK inhibitor.

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REFERENCES