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Ginkgolic acid as a dual-targeting inhibitor for protein tyrosine phosphatases relevant to insulin resistance

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ABSTRACT

Several protein tyrosine phosphatases (PTPs) that disrupt the insulin-signaling pathway were investigated by siRNAs to identify potential antidiabetic targets. Individual knockdown of PTPN9 and DUSP9 in 3T3-L1 pre-adipocytes increased AMPK phosphorylation, respectively, and furthermore, concurrent knockdown of both PTPN9 and DUSP9 synergistically increased AMPK phosphorylation. Next, 658 natural products were screened to identify dual inhibitors of both PTPN9 and DUSP9. Based on the selectivity and inhibition potency of the compounds, ginkgolic acid (GA) was selected for further study as a potential antidiabetic drug candidate. GA inhibited the enzymatic activity of PTPN9 (K_i = 53 μM) and DUSP9 (K_i = 2.5 μM) *in vitro* and resulted in a significant increase of glucose-uptake in differentiated C2C12 muscle cells and 3T3-L1 adipocytes. In addition, GA increased phosphorylation of AMPK in 3T3L1 adipocytes. In this study, GA as a dual targeting inhibitor of PTPN9 and DUSP9 increased glucose uptake in 3T3L1 and C2C12 cells by activating the AMPK signaling pathway. These results strongly suggest GA could be used as a therapeutic candidate for type 2 diabetes.

1. Introduction

Dual and multi-targeting of pathogenic proteins by single drugs has been successfully employed in anticancer drug discovery [1]. For example, cabozantinib was approved by the Food and Drug Administration (FDA) as a small molecule that targets both the inhibitors of the tyrosine kinases c-Met and vascular endothelial growth factor receptor 2 and is used for treating patients with progressive medullary thyroid cancer.

Metabolic syndrome is a multifactorial disease characterized by diverse metabolic abnormalities including obesity, diabetes, hyperlipidemia, and atherosclerosis [2,3]. Type 2 diabetes is a general form of diabetes characterized by an increase in cellular insulin resistance despite normal insulin production by the pancreas. Patients with type 2 diabetes have high risks of serious eye, kidney, nerve, and vascular diseases [4,5]. Recent efforts to discover antidiabetic drugs that target multiple proteins have been reported [6,7].

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) reversibly and coordinately control cellular protein tyrosine phosphorylation levels. The signaling pathways they control are important for a wide variety of cellular responses, such as growth, differentiation, migration, survival, and apoptosis [8,9]. Hence, dysregulation of protein tyrosine phosphorylation is a major cause of human diseases that include cancers, diabetes, autoimmune disorders, and neurological diseases [10].

Several PTPs, including PTPN1, PTPN9, PTPN11, PTPRF, PTPRS, and DUSP-9, have been reported to disrupt the insulin-signaling pathway [10–13]. Protein tyrosine phosphatase, non-receptor type 1 (PTPN1, also named PTP1B) has been shown to downregulate insulin signaling by dephosphorylating insulin receptor and insulin receptor substrates [10]. Inhibition of protein tyrosine phosphatase, non-receptor type 9 (PTPN9, also named PTP-MEG2) results in insulin sensitization and improves glucose homeostasis in diet-induced obese mice, suggesting a potential use of PTPN9 inhibitors for the treatment of diabetes [14]. In addition, dual specificity phosphatase 9 (DUSP9, also named MKP-4) has been shown to negatively regulate insulin signaling and therefore overexpression of DUSP9 in adipocytes inhibits insulin-stimulated glucose uptake [15].

In this study, PTPs were investigated by siRNAs to identify PTPN9 and DUSP9 as potential antidiabetic targets. Next, natural products library was screened for inhibitors of PTPN9 and DUSP9, identifying ginkgolic acid (GA) as a potential antidiabetic drug which targets the both PTPs at the same time. In addition, cell-based studies demonstrated that GA is a dual-targeting therapeutic candidate for the treatment of type 2 diabetes.

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2. Experimental section

2.1. Cell culture

C2C12 muscle cells and 3T3-L1 preadipocytes were obtained from the Korean Cell Line Bank and were cultured as previously described [4,16]. 3T3-L1 preadipocytes were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific Korea Ltd., Seoul, Korea) supplemented with 10% bovine calf serum (BCS; Thermo Fisher Scientific Korea Ltd., Seoul, Korea) and antibiotic-antimycotic solution (Gibco BRL, Middlesex, UK). C2C12 muscle cells were cultured in DMEM supplemented with 15% fetal bovine serum (FBS; Thermo Fisher Scientific Korea Ltd., Seoul, Korea) and antibiotic-antimycotic solution. To assess cell viability, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT; Merck KGaA, Darmstadt, Germany) was used according to the manufacturer's instructions.

2.2. Cell differentiation

The methods used for differentiating 3T3-L1 preadipocytes and C2C12 cells have been described previously [4,16]. When 3T3-L1 preadipocytes reached 100% confluency, they were cultured in DMEM containing 10% FBS, antibiotic-antimycotic solution, 0.5 mM isobutylmethylxanthine (IBMX; Merck KGaA, Darmstadt, Germany), 1 μM dexamethasone (Sigma-Aldrich), and 5 μg/mL insulin (Merck KGaA, Darmstadt, Germany) for 2 days (days 0–2). Cells were then maintained in DMEM supplemented with 10% FBS, antibiotic–antimycotic solution, and 5 μg/mL insulin for a further 2 days (days 3–4) followed by culture in DMEM containing 10% FBS and antibiotic-antimycotic solution for an additional 4 days (days 5–8). When C2C12 muscle cells reached 100% confluency, they were cultured in DMEM supplemented with 2% horse serum (Thermo Fisher Scientific Korea Ltd., Seoul, Korea), antibiotic-antimycotic solution, and 5 μM insulin for 4 days.

2.3. Glucose uptake assay

The methods used for measuring glucose-uptake in C2C12 muscle cells and 3T3-L1 preadipocytes have been described previously [4]. Differentiated cells were cultured in low-glucose DMEM (Welgene, Gyeongsan, Korea) for 4 h and then incubated with 2, 10, 20 μM ginkgolic acid (GA) or Rosiglitazone in glucose-depleted DMEM (Welgene, Gyeongsan, Korea) supplemented with 100 nM insulin for 1 h. The cells were treated with the fluorescent glucose indicators, 5 μM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG; Thermo Fisher Scientific Korea Ltd., Seoul, Korea) and Cy3-labeled glucose bioprobe (GB-Cy3) [17], for 30 min. After washing cells with phosphate buffered saline (PBS), the fluorescence intensity (Excitation/Emission = 470/525 nm) was measured using an EVOS FL Imaging System (Thermo Fisher Scientific Korea Ltd., Seoul, Korea).

2.4. Overexpression and purification of recombinant PTPN9 and DUSP9

The human genes of PTPN9 and DUSP9 with both an *N*-terminal MBP and a C-terminal His_6-tag were transformed into *E. coli* Rosetta (DE3) (Merck Millipore, Darmstadt, Germany). Expression of the recombinant PTPN9 or DUSP9 was induced by the addition of 1 mM or 0.1 mM IPTG; cells were grown at 18 °C for 16 h. Cells were harvested by centrifugation (3570 g at 4 °C for 10 min), washed with buffer A (50 mM Tris pH 7.5, 500 mM NaCl, 5% glycerol, 0.05% 2-mercaptoethanol, and 1 mM phenyl-methylsulfonyl fluoride (PMSF)), and then lysed by ultrasonication. After centrifugation (29,820 g at 4 °C for 30 min), the supernatant was incubated with a cobalt affinity resin (TALON®, Takara Korea, Seoul, Korea) on a rocker at 4 °C for 1 h. The resin was then washed with buffer A containing 10 mM imidazole. PTPN9 and DUSP9 were eluted with buffer A supplemented with 100 mM imidazole, and stored at −70 °C.

2.5. Measurement of enzymatic activities and inhibition constant (K_i)

The enzymatic activities of purified PTPN9 and DUSP9 were measured using 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (100 μM), the most widely employed protein tyrosine phosphatase (PTP) substrate, as described previously [18]. To determine the K_M values, PTPN9 and DUSP9 were added to reaction buffer (20 mM Bis-tris pH 8.0 (PTPN9) or 20 mM tris pH 6.0 (DUSP9), 150 mM NaCl, 2.5 mM dithiothreitol (DTT), 0.01% Triton X-100) containing DiFMUP (800, 400, 200, 100, 50, 25, 12.5, 6.25 μM) to a final volume of 100 μl in a 96 well-plate. Fluorescence intensities were measured continuously for 10 min (Excitation/Emission = 355/460 nm) using a Victor™ X4 multi label plate reader (Perkin Elmer, Waltham, MA, USA), and K_M values were determined by Lineweaver-Burk plots. To estimate the inhibition of DUSP9 and PTPN9 by 658 natural compounds, DUSP9 and PTPN9 were added to solutions containing each of the compounds (20 μM) in reaction buffer with DiFMUP ($2 \times K_M$). To identify the K_i values of GA, GA was added to DiFMUP (61, 122, 244, 488 μM) for PTPN9 or DiFMUP (22.5, 45, 90, 180 μM) for DUSP9 in reaction buffer. After addition of the enzymes PTPN9 (10 nM) and DUSP9 (250 nM), enzyme inhibition was estimated by measuring fluorescence intensities and K_i values were acquired using Dixon and Lineweaver-Burk plots. The GA concentration ranges used to measure inhibition of PTPN9 and DUSP9 at a substrate concentration of $2 \times K_M$ were 9.375–150 μM (PTPN9) and 0.375–3 μM (DUSP9).

2.6. Western blotting

Proteins were extracted using a buffer containing 25 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, and protease inhibitor cocktail (Roche Korea, Seoul, Korea). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Merck KGaA, Darmstadt, Germany) using a wet transfer system. Membranes were incubated overnight at 4 °C with primary antibodies; anti-total AMPK, anti-phosphorylated AMPK (Thermo Fisher Scientific Korea Ltd., Seoul, Korea), and anti-beta-actin (AbFrontier, Seoul, Korea). Membranes were then probed with anti-rabbit-IgG-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Antibody–antigen complexes were detected using an ECL method (GE Healthcare Korea, Songdo, Korea).

2.7. RNA interference

Knockdown of DUSP9 and PTPN9 in 3T3-L1 preadipocytes was performed using small interfering RNAs (siRNAs, Genolution Pharmaceuticals Inc., Seoul, Korea). Scramble siRNA was used as the negative control (Genolution). Transfections were performed using Dharmafect (Dharmacon, GE Healthcare Korea, Songdo, Korea) according to the manufacturer's instructions. The efficiency of DUSP9 and PTPN9 knockdown was measured by quantitative real time- polymerase chain reaction (qRT-PCR).

2.8. Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from 3T3-L1 preadipocytes using RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with DNase (Qiagen) to remove genomic DNA. The total RNA (1 μg) was used to synthesize