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The insulin-mimetic effect of Morin: A promising molecule in diabetes treatment

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ABSTRACT

Background: Type-2 diabetes is a worldwidely diffuse disease characterized by insulin resistance that arises from alterations of receptor and/or post-receptor events of insulin signalling. Studies performed with PTP1B-deficent mice demonstrated that PTP1B is the main negative regulator of insulin signalling. Inhibition or down regulation of this enzyme causes enhanced insulin sensitivity. Hence this enzyme represents the most attractive target for development of innovative anti-diabetic drugs.
Methods: Selection of new PTP1B inhibitors among an in house library of polyphenolic compounds was carried out screening their activity. The inhibition mechanism of Morin was determined by kinetic analyses. The cellular action of Morin was assayed on HepG2 cells. Analyses of the insulin signalling pathways was carried out by Western blot methods, glycogen synthesis was estimated by measuring the incorporation of [^{3}H]-glucose, gluconeogenesis rate was assayed by measuring the glucose release in the cell medium. Cell growth was estimated by cell count. Docking analysis was conducted with SwissDock program.
Results: We demonstrated that Morin: i) is a non-competitive inhibitor of PTP1B displaying a K_i in the μM range; ii) increases the phosphorylation of the insulin receptor and Akt; iii) inhibits gluconeogenesis and enhances glycogen synthesis. Morin does not enhance cell growth.
Conclusions: We have identified Morin as a new small molecular non-competitive inhibitor of PTP1B, which behaves as an activator and sensitizer of the insulin receptor stimulating the metabolic pathways only.
General significance: Our study suggests that Morin is a useful lead for development of new low Mr compounds potentially active as antidiabetic drugs.

1. Introduction

Diabetes mellitus poses a major health problem on both clinical and social plan, not only for the high number of patients, but also for the onset of serious disabling complications that frequently appear. More than 220 million people worldwide are affected by diabetes and its incidence is expected to increase to 400 million by 2030 [1,2]. About 95% of diabetic patients suffer from type-2 diabetes. The onset of this disease is sustained by insulin resistance that arises from alterations of receptor and/or post-receptor events of insulin signalling. Insulin induces autophosphorylation of its receptor and thereby triggers kinase signalling cascades that led to mitosis or produce metabolic effects, such as the stimulation of glycogen and lipid syntheses in the liver or the translocation of GLUT4 onto the plasma membrane of muscle and adipose cells.

Reversible protein tyrosine phosphorylation is one of the strategies most used by eukaryotes to implement the transmission of signals induced by hormones, and growth factors. Both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are involved in balancing the levels of protein tyrosine phosphorylation; regulation of their activities is a very important task for the correct signal progression. Studies performed with substrate-trapping mutants demonstrated that PTP1B can recognize the phosphorylated insulin receptor as a cellular substrate [3]. Other studies performed with PTP1B-deficent mice have revealed phenotypes of enhanced insulin sensitivity, improved glycemic control, and resistance to high fat diet induced obesity [4,5], suggesting that PTP1B is the main negative regulator of insulin signalling. The notion that inhibition of PTP1B should contrast insulin resistance has made this enzyme the most attractive drug target. In fact, several PTP1B inhibitors have been proposed as potential antidiabetic drugs, but the identification of selective and clinically useful PTP1B inhibitors has proven to be extremely difficult since it is often challenged by limited inhibitor selectivity and low bioavailability [6–9]. The difficulty to achieve selectivity for a particular PTP is due to the fact that the human genome encodes more than 100 PTPs, many of which play critical roles as positive or negative modulators of specific signalling pathways in several cellular processes. However, the specific structural characteristics in the loops surrounding

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the active site of PTP1B can be exploited to search or to design inhibitors with sufficient activity and selectivity.

In recent years, there has been a growing interest in anti-diabetic agents from natural products. They represent an alternative mode for diabetes therapy because most of the anti-diabetic drugs have some side effects and fail to significantly alter the course of the disease. In this work we have assayed several polyphenols and related molecules, and found that Morin, a compound yet studied for its anti-cancer activity, possesses a potential anti-diabetic activity. In fact, we found that, in HepG2 cells, Morin displayed a concentration-dependent insulin-mimetic activity through a ligand independent stimulation of insulin receptor that, in turn, promotes glycogen synthesis and gluconeogenesis inhibition.

2. Materials and methods

2.1. Chemicals and reagents

General reagents and culture media were purchased from Sigma-Aldrich. Recombinant human insulin was from Eli Lilly and Co. [^{3}H] D-glucose was purchased from Perkin-Elmer. Antibodies: anti p-IRβ (Y 1162–1163), anti-phosphorylated Akt, anti-phosphorylated FOXO1, anti-phosphotyrosine, and anti-actin were from Santa Cruz Biotechnology Inc. Cell lines: HepG2 (human hepatocyte carcinoma cells, which express constitutively both PTP1B and insulin receptor), NIH-3T3 (mouse embryonic fibroblast cells), and PC3 (human prostate adenocarcinoma cells) were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK.

2.2. Recombinant PTPs

The complete coding sequences of human IF1, IF2 LMW-PTPs, yeast Ltp1, and human PTP1B were cloned in frame with the sequence of the glutathione S-transferase in the pGEX-2T bacterial expression vector. Enzyme expressions were achieved in the *Escherichia coli* TB1 strain, and each PTP was purified as briefly described. The recombinant fusion proteins were purified from bacterial lysate using a single step affinity chromatography on glutathione-Sepharose. The solution containing purified fusion proteins was treated with thrombin for 3 h at 37 °C. Then the enzymes were separated from GST and thrombin by gel filtration on Superdex G-75. The purity of protein preparations was analyzed by SDS–PAGE according to Laemmli. [10]. Recombinant TC-PTP and YopH-PTP were obtained from New England Biolabs and from Calbiochem, respectively.

2.3. Phosphatase assay and inhibition experiments

Phosphatase assay was carried out at 37 °C using *p*-nitrophenylphosphate (*p*NPP) as substrate. The assay buffer contained 0.075 M sodium β,β-dimethylglutarate buffer, pH 7.0, and 1 mM EDTA. The final volume of the assay mixture was 1 ml. The reactions were initiated by addition of aliquots of enzyme preparations and stopped at appropriate times by adding 4 ml of 1 M KOH. The released *p*-nitrophenolate ion was determined by reading the absorbance at 400 nm ($\varepsilon = 18{,}000\ M^{-1}\ cm^{-1}$).

The IC_{50} values for the PTP inhibitors were determined by measuring the initial hydrolysis rate under fixed *p*-NPP concentration, equal to the K_m value of each tested PTP. Data were fitted to the following equation using the FigSys program:

$$y = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{IC_{50}}\right)^{\text{slope}}} + \text{Min}$$

where $y = v_i/v_o$, i.e. the ratio between the activity measured in the presence of the inhibitor (v_i) and the activity of the control without the inhibitor (v_o). The parameter "*x*" is the inhibitor concentration. The main kinetic parameters (K_m and V_{max}) were determined by measuring the initial rates at different substrate concentrations. Experimental data were analysed using the Michaelis equation and a non-linear fitting program (FigSys). Inhibition constants were determined measuring initial hydrolysis rates at differing substrate and inhibitor concentrations. The apparent K_m values, measured at the various inhibitor concentrations, were plotted against concentration of the inhibitor to calculate the K_i values. All measurements of initial rate were carried out in triplicate.

To verify if Morin and Apigenin were reversible inhibitors, appropriate aliquots of PTP1B were incubated in the presence of at 50 μM or 100 μM of inhibitor for 1 h at 37 °C. Control experiments were performed adding DMSO instead of inhibitor. After this interval time, the enzyme solutions were diluted 400-fold and the residual enzyme activity was assayed.

2.4. Docking

Docking calculations were conducted with SwissDock, a protein-small molecule docking web service based on EADock DSS (Fast docking using the CHARMM force field with EADock DSS [11]. SwissDock is based on the docking software EADock DSS, whose algorithm consists of the following steps: i) many binding modes are generated either in a box (local docking) or in the vicinity of all target cavities (blind docking); ii) simultaneously, their CHARMM energies are estimated on a grid; iii) the binding modes with the most favorable energies are evaluated with FACTS, and clustered; iv) the most favorable clusters can be visualized online and downloaded on a personal computer (http://swissdock.vital-it.ch/). UCSF Chimera (a visualization system for exploratory research and analysis) was used for molecular structure viewing [12]. The crystal structure was obtained from the PDB with the accession code 2QBS [13]. Morin 3D structure was obtained by the Zinc Data base [14] (code 3881558, ZINC 12 Beta release Dec. 1, 2011. Production Release Jan. 1, 2012).

2.5. Cell culture

HepG2, NIH-3T3, and PC3 cells were grown in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 humidified atmosphere. Before treatments cells were starved for 20 h without FBS, and then Morin, insulin or both Morin and insulin, were added to the culture medium; incubation was performed at 37 °C for the appropriate times. Control experiments were performed adding DMSO. Cells were then lysed with the SDS-Laemmli sample buffer and analyzed by SDS-PAGE/Western Blot using the specific antibodies indicated in each particular experiment.

2.6. Stimulation of glucose incorporation into glycogen

Glycogen synthesis was assayed as follows: Immediately after treatment with Morin, with insulin or with both Morin and insulin, [^{3}H]-glucose in Ringer–Hepes buffer was added to each well (0.5 μCi/ml final concentration). Glucose uptake was allowed at 37 °C for 120 min, when the cells were washed three times with ice-cold phosphate-buffered saline. They were then solubilized with 2 M NaOH. After 30 min cold ethanol was slowly added to the lysates with stirring to reach the final concentration of 66% (v/v); then samples were left overnight at −20 °C. Finally, samples were centrifuged at 18,000 ×*g* for 30 min, the precipitates were dissolved in water, and an appropriated aliquot of each sample was diluted with the aqueous scintillation fluid, and counted. Data were normalized against protein concentration.