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Original article

Establish a dipeptidyl peptidases (DPP) 8/9 expressing cell model for evaluating the selectivity of DPP4 inhibitors

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ABSTRACT

Introduction: Dipeptidyl peptidases (DPPs) 8 and 9 are homologous, cytoplasmic postproline-cutting enzymes, which have similar enzymatic activity and preferred substrates as DPP4. DPP4 is a well-known target for treating diabetes mellitus. With the increased concern of non-selectivity and toxicities caused by DPP4 inhibitors, it is essential to establish new ex vivo system to investigate DPP4 inhibitors' effect on DPP8 and DPP9.
Method: Here we reported a newly established cell model system by cloning and transfecting human DPP8/9 genes into HEK 293 cells. We then used this model to evaluate the clinically applied DPP4 inhibitors' effect on DPP8/9, by direct enzymatic activity assay. Given the difference of cellular locations between DPP4 and DPP8/9, we also evaluated the influence of these drugs on intracellular DPP8/9 activity and cell viability by extracellular treatment with different inhibitors.
Results: Direct enzymatic activity assay revealed significant and concentration-dependent inhibition effect of vildagliptin, saxagliptin on DPP8/9. Extracellular incubation of DPP8/9 over expressed cells with sitagliptin, vildagliptin, saxagliptin, alogliptin and linagliptin, showed only mild inhibition on DPP8/9. Moreover, all of these drugs showed no significant influence on cell viability.
Discussion: Our results demonstrated that the DPP8/9 over-expressing cell model system is a very useful and promising system for investigating the selectivity and associated toxicity of DPP4 inhibitors on DPP8/9.

1. Introduction

The increasing worldwide prevalence of diabetes mellitus, especially type 2 diabetes mellitus, calls for new and effective treatment strategies to be developed and implemented. Recently, dipeptidyl peptidase (DPP) 4 inhibitors have emerged as a new class of drugs for diabetes therapy. DPP4 inhibitors can prevent the enzymatic degradation of endogenous glucagon-like peptide (GLP)-1, a well-known peptide that can stimulate insulin secretion from the islet β-cells which can also stimulate proliferation and concurrently suppress β-cell apoptosis (Deacon, 2011). Furthermore, the potential effects of DPP4 inhibitors may take in some benefits to diabetes-associated syndrome such as cardiovascular and chronic kidney disease (Aroor et al., 2013). In addition, DPP4 inhibitors have been reported as an emerging drug family targeting inflammatory disease (Yazbeck, Howarth, & Abbott, 2009).

DPP4 belongs to the S9b gene family of postproline serine peptidases. It acts mostly as a secreted membrane protein, mediating the degradation and inactivation of GLP-1 and gastric inhibitory protein (GIP). Besides DPP4, there are many other DPPs, such as DPP2, 6, 8, 9 and 10 (Qi, Riviere, Trojnar, Junien, & Akinsanya, 2003). In this study, we only focused on DPP8 and DPP9. The gene that encodes DPP8 is located on the human chromosome locus 15q22, produces an 882-amino-acid long cytosolic protein (Abbott, Woollatt, Sutherland, McCaughan, & Gorrell, 2000). The DPP9 gene is located at the human chromosome locus 19p13.3, which encodes two variants, an 863-amino-acid and an 892-amino-acid long cytosolic protein (Ajami, Abbott, McCaughan, & Gorrell, 2004). Previous studies by using recombinant expression systems demonstrated that DPP8 and DPP9 can successfully cleave identified DPP4 substrates, such as neuropeptide Y(1–36), glucagon-like peptide-1(7–36), glucagon-like peptide-2(1–33), and peptide YY(1–36) (Bjelke et al., 2006). It is also known that DPP8 can cleave some chemokines, such as inflammatory protein-10(IP10), interferon-inducible T cell chemo-attractant(ITAC), and stromal cell-derived factors 1a and 1b (SDF-1a and 1b) (Ajami et al., 2008). DPP9 is reported to involve in degradation of proline-containing peptides, such as $RU1_{34-42}$ antigenic peptide (Geiss-Friedlander et al., 2009). Recently, calreticulin and adenylate kinase 2 have been identified as two cytosolic substrates for DPP8 and DPP9, which suggest the functions of DPP8 and DPP9 in cellular homeostasis and energy metabolism (Wilson et al., 2013). These data inspired us to re-evaluate the role of DPP8 and DPP9 both in vivo and ex vivo and explore the difference between DPP8/9 and DPP4 inhibition according to their different active forms, cellular locations (Yu et al., 2009) and biological functions (Matheeussen et al., 2013; Yu, Wang, McCaughan, & Gorrell, 2006).

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The importance of selective inhibition of DPP8/9 has driven massive research attention recently as studies identified that some DPP8/9 inhibitors could cause severe toxicities, such as alopecia, thrombocytopenia, reticulocytopenia, enlarged spleen, multiorgan histopathological changes, and increased mortality in a 2-week rat toxicity study and bloody diarrhea in an acute dog tolerability study (Lankas et al., 2005). However, some subsequent researches using other selective DPP8/9 inhibitors, such as UAMC00132 (Maes et al., 2007), 1G244 (Wu et al., 2009) and vildagliptin, demonstrated that the inhibition of DPP8/9 did not cause organ toxicities or death in rodents. Nevertheless, it is believed that the development of DPP4 inhibitors should definitely include an assessment of the inhibition selectivity on DPP8/9 (Burkey et al., 2008).

Currently there are several DPP4 inhibitors on the market such as sitagliptin (launched in 2006), vildagliptin (launched in 2007), saxagliptin (launched in 2009), alogliptin (launched in 2010) and linagliptin (launched 2011). However, some drugs are not specific for targeting DPP4 (Chen & Jiaang, 2011), while they did not cause significant toxicities associated with DPP8/9 inhibition in clinical applications (Kirby, Yu, O'Connor, & Gorrell, 2010). In this work, we successfully established a DPP8/9 over-expressing cell model system by transient transfection of DPP8/9 genes into HEK 293 cells, and we then evaluated the inhibition effect of the commercial available drugs on DPP8/9 and cell viability by direct enzyme activity assay as well as extracellular incubation of these drugs with the DPP8/9 over-expressing cells.

2. Materials and methods

2.1. Chemicals and reagents

For the DPP inhibitor candidates, sitagliptin, vildagliptin, and saxagliptin were purchased from Beijing Huikang Boyuan Chemical Tech. Co. Ltd (China), UAMC00132, alogliptin and linagliptin were synthesized by chemical laboratory in house, all the compounds were dissolved in DMSO and added into the enzymatic assay or cell culture medium at a final DMSO concentration of 0.1%. Dulbecco's modified Eagle's medium (DMEM), Lipofectamine™ 2000, Trizol reagent were purchased from Life Technologies (USA). Fetal bovine serum (FBS) was purchased from Biochrome AG (Germany). All the cell plates were products of Corning-Costar (USA). First-strand cDNA synthesis kit was purchased from Vigorous (Beijing, China). Restriction enzymes were purchased from Takara (Japan). The synthetic substrate of DPP enzyme, Gly-Pro-p-nitroanilide, was purchased from Sigma Aldrich and then diluted to the appropriate concentration in the Tris–Cl buffer (PH8.0). Protein quantity BCA assay kit was purchased from Applygen Technologies (Beijing, China). Antibodies targeting human DPP4/8/9 (TA500733/TA504366/TA504040) and β-actin (TA310155) were purchased from Beijing OriGene Technology Inc. To confirm DPP4 inhibitory effect of all compounds and quality of DPP4 antibody, the purified recombinant human DPP4 protein was purchased from Sino Biological Inc. (Beijing, China), and used in enzymatic reaction and Western blot as positive controls respectively. MTT stock solution was prepared by dissolving 5 mg MTT (thiazolyl blue tetrazolium blue, M2128, Sigma Aldrich, USA) into 1 ml phosphate buffer saline and filtered at 0.22 μm to sterilize and remove insoluble substances.

2.2. Cloning and construction of recombinant human DPP8 and DPP9

The total RNA was extracted from HeLa and HepG2 cells using Trizol reagent and reverse-transcription (RT)-PCR was performed to get the full length of human DPP8 and DPP9 cDNA fragments, respectively. Primers for human DPP8 (GenBank ID: AF221634; 5′-ATCAAGCTTGCC ACCATGGCAGCAGCAATG-3′ and 5′-ATCGTCGACTTATATCACTTTTAGAG CAGCAATACG-3′) and DPP9 (GenBank ID: AF374518; 5′-ATCGGATCCG CCACCATGGGGAAGGTTAAG-3′ and 5′-ATCCTCGAGTCAGAGGTATTCC TGTAGAAAGTGCAG-3′) were designed with additional restriction enzyme sites for cloning. The PCR products of human DPP8/9 (hDPP8/9) were separately cloned into the pcDNA3.1-(+) vector, and confirmed by sequencing.

2.3. Eukaryotic protein expression and identification

293E cells were seeded in 6-well plates and cultured to 60–70% confluence for 24 h, then the cells were transiently transfected with pcDNA3.1, pcDNA3.1-hDPP8 or pcDNA3.1-hDPP9 (2 μg, respectively) using Lipofectamine™ 2000, following the recommended protocol. After 48 h of transfection, cells were collected and lysed with 20 mM Tris–Cl buffer (PH8.0, added with 1% Nonidet P-40, 1% Sodium deoxycholate, 40 mM KCl). Enzymatic activity in the lysate was determined and represented as the increased OD value at the wavelength of 405 nm after incubation of 100 μg of lysates and 0.26 mM Gly-Pro-p-nitroanilide in 100 μl Tris–Cl buffer system for 40 min. The activity of hDPP8/9 was normalized to the percent enzymatic activity in lysate of the mock-transfected cells. Meanwhile, using specific antibodies, the protein levels of DPP4/8/9 in the lysates were analyzed by Western blot.

2.4. Evaluate the inhibitory potency of DPP4 inhibitors based on DPP8/9 enzymatic activity assay

To examine the inhibitory effect of drugs on human DPP8/9, the hDPP8/9 over-expressing cells were lysed and 100 μg of lysate was added in the enzymatic activity assay. Within 100 μl of the final volume, the final concentration of DPP inhibitors used in the assay was 10 μM, DMSO at the concentration of 0.1% was applied as a solvent and negative control, and the final concentration of Gly-Pro-p-nitroanilide was 0.26 mM. The OD value was detected immediately before and after 40 min of reaction at 37 °C, and then enzymatic activity was calculated. The percentage of hDPP8/9 activity was calculated by the following equation: $\Delta OD_{drug} / \Delta OD_{control} \times 100\%$. To assess the concentration–

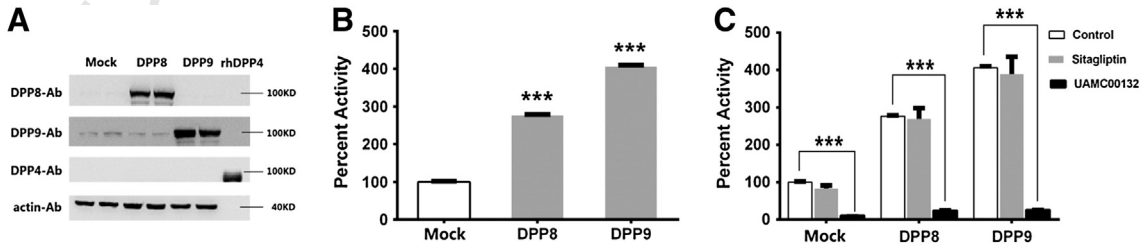

Fig. 1. A. Western blot analysis of protein levels of DPP4/8/9 in 293E cells transfected with pcDNA3.1 (Mock), pcDNA3.1-hDPP8 (DPP8) and pcDNA3.1-hDPP9 (DPP9), the purified recombinant human DPP4 protein (rhDPP4) was used as a positive control for the DPP4 antibody. B. DPP enzymatic activities in 293E cells transfected with pcDNA3.1 (Mock), pcDNA3.1-hDPP8 (DPP8) and pcDNA3.1-hDPP9 (DPP9) were determined, ***$p < 0.001$ compared to mock-transfected group. C. The inhibitory effects of two DPP inhibitors, sitagliptin and UAMC00132, to the DPP enzyme in the lysates of 293E cells transfected with pcDNA3.1 (Mock), pcDNA3.1-hDPP8 (DPP8) and pcDNA3.1-hDPP9 (DPP9) were investigated; the final concentration of sitagliptin and UAMC00132 was 10 μM,***$p < 0.001$ compared to indicated control, respectively.

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