European Journal of Pharmacology 771 (2016) 65–76

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

Hepatic role in an early glucose-lowering effect by a novel dipeptidyl peptidase 4 inhibitor, evogliptin, in a rodent model of type 2 diabetes

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ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form
13 November 2015
Accepted 18 November 2015
Available online 24 November 2015

Keywords:
Dipeptidyl peptidase-4
Evogliptin
TORC2
Liver
PDX-1
Pancreas

Chemical compounds studied in this article:
Evogliptin (PubChem CID: 25022354)
Streptozotocin (24899428)

ABSTRACT

Although multiple dipeptidyl peptidase 4 (DPP4) inhibitors have shown glucose-lowering effects by preserving pancreatic cells in high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice, the hepatic role in regulation of glucose homeostasis by DPP4 inhibitors in HFD/STZ mice remains elusive. In herein study, parallel comparison of effects on the liver (expression of gluconeogenic genes and the linked signaling molecules) and pancreas (islet morphology and relative area of alpha or beta cells) in combination with glucose-lowering effects were made at the end of 2- and 10-week of evogliptin treatment in HFD/STZ mice. Significant control of hyperglycemia was observed from the second week and persisted during 10-week treatment of 0.3% evogliptin in HFD/STZ mice. This effect was accompanied by increased level of plasma glucagon-like peptide-1 and preserved pancreas islet structure. Furthermore, the hepatic increases in gluconeogenic gene expression in HFD/STZ mice was significantly reduced by evogliptin treatment, which was accompanied by the suppression of cAMP response element-binding protein (CREB) phosphorylation and expression of transducer of regulated CREB protein 2. This hepatic effect of evogliptin treatment was reproduced in 2-week study, however, pancreatic beta-cell area was not altered yet although the expression of pancreatic and duodenal homeobox protein 1 was increased. We conclude that the suppression of hepatic gluconeogenesis by evogliptin is followed by preservation of pancreatic islet, leading to remarkable and persistent glucose-lowering effect in HFD/STZ mice. Our findings provide further insight for the hepatic role in DPP4 inhibitor-mediated glucose control in diabetes.

1. Introduction

Dipeptidyl peptidase 4 (DPP4; EC 3.4.14.5) is a ubiquitously distributed serine protease (Heike et al., 1988; Dinjens et al., 1989; Lamers et al., 2011), which is known to be involved in the regulation of various endogenous peptides including glucagon-like peptide-1 (GLP-1). DPP4 inhibitors have pleiotropic pharmacological functions such as attenuation of fatty liver, liver fibrosis and obesity-induced adipose tissue inflammation in addition to pancreatic protection (Mu et al., 2006; Shirakawa et al., 2011; Itou et al., 2012; Yilmaz et al., 2012; Zhong et al., 2013), while glucose-lowering activity by DPP4 inhibition is mainly pancreatic effects by the prolonged action of endogenous GLP-1 (Mannucci et al., 2005; Vilsboll et al., 2001).

Type 2 diabetes (T2D) manifests hyperglycemia caused by peripheral insulin resistance and the disturbance of insulin secretion. High-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice are characterized by severe hyperglycemia, hyperlipidemia, insulin resistance and liver steatosis (Luo et al., 1998; Mu et al., 2009). The pharmacological efficacies of several DPP4 inhibitors have been tested in the HFD/STZ mice model, where DPP4 inhibitors reached sub-maximal efficacy within 2–4 weeks of administration (Mu et al., 2006, 2009; Zhang et al., 2011). Glucose

Abbreviations: AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; AUC, area under the curve; CREB, cAMP response element-binding protein; DPP4, dipeptidyl peptidase 4; eWAT, epididymal white adipose tissue; FOXO1, forkhead box protein O1; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; *G6pc*, glucose-6-phosphatase; HbA1c, glycated hemoglobin; HCl, hydrochloric acid; HFD, high-fat diet; ITT, insulin tolerance test; NEFA, non-esterified fatty acids; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PDX-1, pancreatic and duodenal homeobox protein 1; *Pepck*, phosphoenolpyruvate carboxylkinase; PKA, protein kinase A; *Ppargca1a*, peroxisome proliferator-activated gamma coactivator 1α; qPCR, real time quantitative polymerase chain reaction; S.D., standard deviation; S.E.M., standard error of the mean; STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes; TG, triglycerides; TORC2, transducer of regulated CREB protein 2

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http://dx.doi.org/10.1016/j.ejphar.2015.11.029
0014-2999/

control can be the integrated response of DPP4 inhibitors to pancreas, liver, skeletal muscle, adipose tissue etc. However, previous studies have mainly focused on the chronic glucose control including preservative effects on pancreas in HFD/STZ mice, while there are no reports on extra-pancreatic effects of DPP4 inhibitors in glycemic control of HDF/STZ mice. In addition, the pancreatic impairment following STZ injection continuously progress, which implies the different disease state at different time points. Therefore it is worth exploring time-differential effects and extra-pancreatic effects of DPP4 inhibitors in HFD/STZ diabetic mice.

In this study, we used evogliptin, a potent and selective inhibitor of DPP4 which just got an approval from Korean Ministry of Food and Drug Safety for the treatment of T2D (Kim et al., 2012). Evogliptin has been found to lower blood glucose levels in both type 1 and type 2 genetic or diet-induced hyperglycemia models (Cho et al., 2011; Kim et al., 2012). Here, we assumed that the hyperglycemia of HFD/STZ mice may be resulted from different etiology at early and late phase. We tried to determine time-differential effects of evogliptin and to dissect extra-pancreatic functions from the pancreatic effect of evogliptin on glucose control using pre-screened HFD/STZ mice with hyperglycemia, hypertriglyceridemia, and insulin resistance for 2-week and 10-week administration.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich, St Louis, USA.

2.2. Generation of HFD/STZ mice

This study was conducted in compliance with Korean legislation under the Laboratory Animal Act 2009. Animal experiments were approved by the Institutional Animal Care and Use Committee of Dong-A ST Research Institute (I-1307050, I-1312031).

Four-week-old male ICR mice were purchased from Dae Han Biolink (Eumsung, Korea) and the mice were maintained under a controlled environment, with temperature at 23 ± 2 °C, relative humidity at $55 \pm 5\%$ and a 12-h/12-h light/dark cycle throughout the experiment. Two mice were housed in a cage fed on either a HFD (D12492, Research Diets, New Brunswick, NJ; 60% of calories from fat) or a normal diet. After a 3-week HFD feeding, a single dose of STZ (80 mg/kg in 0.1 M citrate buffer, pH 4.5) was administered by intraperitoneal injection to induce partial insulin deficiency (Luo et al., 1998; Mu et al., 2009). Three weeks after the STZ injection, the majority of animals fed a HFD and treated with STZ exhibited hyperglycemia. The HFD/STZ mice with distinct hyperglycemia concomitant with insulin resistance and hypertriglyceridemia (beyond $1 \sim 2$ S.D. values from the normal mice mean) were then selected for subsequent studies. Based on blood glucose, glycated hemoglobin (HbA1c), insulin resistance assessed by ITT, and plasma triglycerides levels, HFD/STZ mice were randomly allocated into three groups (Table S1).

2.3. Drug treatment

In-house synthesized evogliptin ((R)-4-[(R)-3-amino-4-(2, 4, 5-trifluorophenyl)butanoyl]-3-(t-butoxymethyl) piperazin-2-one, purity > 98.0%) L-tartrate salt (Kim et al., 2011) was provided to mice as the drug-diet admixture (0.1% and 0.3% (w/w) for 100 mg/kg/day and 300 mg/kg/day) for 2 or 10 weeks. The relatively high doses of evogliptin were necessary to maintain effective drug levels for 24 h due to low bioavailability (50 ng h/ml of $AUC_{0-24\,h}$ at 1 mg/kg per os) and short half-life (2–3 h) of evogliptin especially in mice. The doses were selected based on the previous experiments showing that single administration of 100 mg/kg and 300 mg/kg evogliptin led to 65% and 87% inhibition of plasma DPP4 activity at 24 h-postdose in mice. Fed blood glucose level, body weight, and food intake were monitored weekly in the morning. The change in HbA1c levels was measured on weeks 0, 4, and 10 using DCA 2000+ (Bayer Healthcare, Wuppertal, Germany). After treatment, the mice were sacrificed without fasting. Blood was collected separately in heparinized tubes with/without a DPP4 inhibitor (sitagliptin). The liver, pancreas, and epididymal fat pad (eWAT) were isolated and immediately frozen. The isolated pancreas was immersed in phosphate-buffered saline (PBS) containing 10% formaldehyde for histology.

2.4. Oral glucose tolerance tests (OGTT)

On week 8, 4 h-fasted mice were orally challenged with a glucose solution (2 g/kg) and blood glucose levels were measured at the indicated time points in tail vein blood using a glucometer (AccuChek Active, Roche Diagnostics, Mannheim, Germany).

2.5. Insulin tolerance tests

On week 9, 6 h-fasted mice were intraperitoneally injected with insulin (0.75 U/kg) and blood glucose levels were measured as described above. The blood glucose excursion as a percentile of the baseline blood glucose over 2 h was presented as AUC ($AUC_{0-2\,h}$, % min).

2.6. Biochemical parameters

Insulin (Shibayagi, Gunma, Japan), glucagon (Yanaihara, Shizuoka, Japan), active GLP-1 (Linco Research, St Charles, USA), non-esterified fatty acids (NEFA; Wako Pure Chemical Industries, Osaka, Japan) and plasma triglycerides (TG) (Wako Pure Chemicals, Osaka, Japan) were determined using enzymatic assay kits. Liver TG was extracted according to the following process. The isolated liver tissue ($\sim$80 mg) was homogenized in 1 ml distilled water containing 5% Triton X-100, slowly heated to 80–100 °C in a water bath for 5 min, then cooled to room temperature. This heating-cooling cycle was repeated once. The samples were centrifuged at 10,000 xg for 2 min at 4 °C and the supernatants were used for quantification of TG using a colorimetric kit (Biovision, Milpitas, CA). Liver TG content was presented as the relative amount of TG per tissue weight used.

2.7. Plasma DPP4 activity

After 2-week treatment, mice were sacrificed without fasting and plasma was separated. Fifty microliters of plasma was added to the same volume of an assay buffer (100 mM HEPES, pH 7.6, 0.1 mg/ml bovine serum albumin) containing 50 μM Gly-Pro-7-amido-4-methylcoumarin (AMC) as a substrate (Bachem, Torrance, CA). The fluorescence intensity of AMC released by the enzymatic activity was monitored every 20 s for 5 min using an Infinite M1000 (TECAN, Grödig, Austria) with 360 nm excitation and 465 nm emission filters.

2.8. Pancreatic insulin content

The isolated pancreas homogenized in ice-cold acidic ethanol (0.15 mol/l hydrochloric acid (HCl) in 75% (v/v) ethanol), was incubated overnight at −20 °C. After centrifugation, the supernatants were neutralized using 1 mol/l Tris buffer (pH 7.5). The pancreatic extracts were used for quantifying insulin (Shibayagi,