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Additive effects of evogliptin in combination with pioglitazone on fasting glucose control through direct and indirect hepatic effects in diabetic mice

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ABSTRACT

Due to very limited preclinical reports, pharmacodynamic interactions between dipeptidyl peptidase 4 (DPP4) inhibitors and peroxisome proliferator-activated receptor γ (PPARγ) agonists are not conclusive yet. This study aimed to evaluate the pharmacological responses from adding evogliptin, a DPP4 inhibitor, to pioglitazone, a PPARγ agonist, in diabetic db/db mice after a 2-week treatment. This combination led to further decrease in both fasting and fed blood glucose levels compared to evogliptin alone ($P < 0.05$), but combination effects were more dramatic in fasting glucose levels ($P < 0.05$ vs. each treatment alone). Of note, plasma glucagon and high-molecular-weight (HMW) form of adiponectin were also further altered by the combination ($P < 0.05$ vs. each treatment alone). In line with these results, hepatic gluconeogenic gene expression was normalized by this combination. However, although evogliptin or pioglitazone directly suppressed glucose output in HepG2 hepatocytes, their combination did not further reduce hepatic glucose output. By contrast, glucose utilization of HepG2 cells was synergistically enhanced by this combination regardless of insulin presence ($P < 0.05$ vs. each treatment alone). These results suggest that the combination of evogliptin and pioglitazone is more efficacious in fasting glucose control through systemic alterations such as decreasing glucagon and increasing adiponectin, and through enhancing glucose utilization. To our knowledge, this is the first report regarding the significant combination effects of DPP4 inhibitors plus PPARγ agonists on plasma HMW adiponectin and hepatic glucose utilization. Our findings provide insight that the evogliptin and pioglitazone combination therapy may be more beneficial in type 2 diabetic patients characterized by exaggerated glucagon dysregulation.

1. Introduction

Type 2 diabetes is a progressive disease characterized by deficient insulin secretion and impaired insulin action. Therefore drugs that stimulate insulin secretion or improve insulin sensitivity have been developed over the past several decades. Given that diabetes is a complex multifactorial disease, the standard care for type 2 diabetes goes through combination therapies involving two or three drugs with different modes of action when the patient does not respond to metformin monotherapy (Thrasher, 2017). Up to now, several classes of glucose-lowering agents had been developed such as insulin secretagogues, insulin sensitizers, and inhibitors of intestinal absorption or renal reabsorption of glucose, etc. (Mittermayer et al., 2015). Considering the pathophysiological factors, combination treatment with an insulin secretagogue and an insulin sensitizer can be an ideal therapeutic option.

Although sulfonylureas are powerful insulin secretagogues, the risk of hypoglycemia and pancreatic exhaustion has limited their clinical use. Dipeptidyl peptidase 4 (DPP4) is a serine protease that inactivates various endogenous peptide substrates. DPP4 inhibitors block degradation of a biologically active form of glucagon-like peptide-1 (GLP-1) and stimulate insulin secretion through activation of pancreatic GLP-1 receptors. DPP4 inhibitors have moderate hypoglycemic efficacy and have proven to be well tolerated and safe with low hypoglycemia risk over the past decade (Foroutan et al., 2016). Peroxisome proliferator-activated receptor γ (PPARγ) agonists are strong insulin-sensitizing agents, but accumulating evidences have been raised regarding the safety concerns such as heart failure, edema, weight gain, and bladder cancer (Horita et al., 2015; Komajda et al., 2010; Lubet et al., 2008). Thus pharmaceutical companies have attempted to develop safer insulin sensitizers, but a novel class of insulin sensitizer to replace the existing PPARγ agonists has not been available yet.

Previously, it was demonstrated that type 2 diabetic patients on combination therapy with a DPP4 inhibitor and pioglitazone, a PPARγ agonist, had more glycemic control than on either monotherapy. They generally showed a noteworthy reduction of fasting glucose levels in addition to postprandial ones, but no additional improvement of insulin

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resistance from the combination treatment compared to either monotherapy (Gomis et al., 2011; Kaku et al., 2011; Rosenstock et al., 2006). However, few data on biomarkers were available to delineate the modes of action and their pharmacodynamic interactions in detail in the clinical trials reported. Meanwhile, non-clinical results on antidiabetic effects are very limited for studies with the combination of alogliptin and pioglitazone (Cummings et al., 2014; Hirukawa et al., 2015; Kawashima et al., 2011; Moritoh et al., 2009a, 2009b), presumably because Onsei® (Incresync® for Europe) is the only combination tablet with a DPP4 inhibitor and a PPARγ agonist developed to date (Aoki et al., 2017). Because there is a paucity of information examining the pharmacodynamic interactions between DPP4 inhibitors and PPARγ agonists, it remains unclear as to how the combination of DPP4 inhibitors and PPARγ agonists lowers blood glucose more than each drug alone.

In this study, we intended to evaluate the pharmacodynamic interactions of evogliptin, a DPP4 inhibitor approved in 2016 in South Korea, with pioglitazone in diabetic mice.

2. Materials and methods

2.1. Materials

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 synthesized internally and pioglitazone hydrochloride salt was purchased from Aurobindo Pharma (Hyderabad, India). 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was a product of R&D Systems (#6065, Minneapolis, USA). Unless otherwise specified, chemical reagents and antibodies were purchased from Sigma-Aldrich (St Louis, USA) and Cell Signaling Technology (Beverly, USA), respectively.

2.2. db/db mice

This study was performed with approval of the Institutional Animal Care and Use Committee of Dong-A ST Research Institute (permit number: I-1506176) in accordance with the Laboratory Animal Act of the Korean Ministry of Food and Drug Safety.

Male pre-diabetic db/db mice with a C57BL/KsJ genetic background and non-diabetic wild-type mice aged 5 weeks were purchased from Central Lab. Animal Inc. (Seoul, Korea). All mice were maintained at 23 ± 2 °C on a 12:12-h light-dark cycle (lights on 0700–1900 h) and acclimated for 1 week. Then, *db/db* mice were allocated to corresponding groups (8 animals/group) according to body weight, glycated hemoglobin (HbA1c), and fed blood glucose levels, and all mice were group housed with free access to water and normal chow diet (Purina Laboratory Rodent Diet #38057, Cargill Agri Purina, Seoul, Republic of Korea).

2.3. Drug treatment

Evogliptin or pioglitazone alone or combination treatment as suspended in 0.5% (w/v) methylcellulose solution (10 ml/kg) was orally given once daily in the morning for 14 days to *db/db* mice at the age of 6 weeks. Age-matched normal mice (6 animals) and *db/db* control mice (8 animals/group) were treated daily by oral gavage with vehicle (0.5% methylcellulose). Evogliptin was administered at a dose of 200 mg/kg as free form because the minimum daily effective dose maintaining over 80% inhibition of plasma DPP4 activity for 24 h was determined to be at least 100 mg/kg in diet-induced obese mice (Kim et al., 2012). A minimum effective dose (3 mg/kg) and a moderate dose (12 mg/kg) of pioglitazone were each selected according to unpublished internal data and previous reports (Ishida et al., 2004; Makino et al., 2006) to determine the combination effect. During the treatment period, non-fasting blood glucose at 2 h post-dose was measured from the tail vein blood using a glucometer (Roche, AccuChek Active, Ireland) three times per week. HbA1c levels were measured before and after treatment using a DCA2000 + (Bayer Healthcare, Wuppertal, Germany). On day 14, mice were euthanized at 2 h after the last administration without fasting. Blood was collected separately in heparinized tubes with/without a DPP4 inhibitor. The liver was isolated and immediately frozen.

2.4. Insulin tolerance tests

On Day 13, 6 h-fasted mice after drug administration were intraperitoneally injected with insulin (0.75 U/kg), and blood glucose levels were measured at pre-injection, 15, 30, 60, 90, and 120 min after injection. The area under the curve of time-blood glucose level as a percentile of the baseline over 2 h was presented as $AUC_{0-2\,h}$ (%·min).

2.5. Biochemical parameters

Plasma active GLP-1 (EGLP-35K, Millipore, St Charles, USA), glucagon (YII-YK090-EX, Cosmo Bio, Carlsbad, USA) and high-molecular-weight (HMW) adiponectin (AKMAN-011, Shibayagi, Gunma, Japan) were measured using commercially available enzyme-linked immunosorbent assay kits.

2.6. Immunoblotting

Liver tissue was homogenized in RIPA buffer supplemented with EDTA-free complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), 50 mM sodium fluoride, 1 mM sodium orthovanadate and 5 mM sodium pyrophosphate. After centrifugation at 13,000 × *g* for 5 min at 4 °C, total protein in the lysate was determined using a bicinchoninic acid kit (Pierce, Rockford, USA). Thirty micrograms of total protein was separated on a 4–12% precast Bis-Tris gel (Invitrogen, Carlsbad, USA) in MOPS buffer and transferred to PVDF membranes. Non-specific binding was blocked with Tris buffer containing 5% (w/v) skim milk and 0.1% (v/v) Tween-20 at RT for 1 h. The dilutions of antibodies in Tris buffer containing 5% (w/v) bovine serum albumin were 1:1000 and 1:5000 for primary and secondary antibodies, respectively. The antibodies used were as follows: anti-phosphoAkt (473Ser) (#9271), anti-Akt (#9272), anti-beta actin (#4967) and anti-rabbit IgG HRP conjugate (1 mg/ml; #W4011, Promega, Madison, USA). Blots were developed with FEMTOMAX-110 reagents (Rockland Immunochemicals, Gilbertsville, PA, USA), imaged on a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA), and quantified using ImageJ software (NIH, Bethesda, USA).

2.7. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA (1 μg) extracted from the liver tissue with TRIzol® reagent (Invitrogen, Carlsbad, USA) was reverse-transcribed using random hexamers. qPCR was performed as described previously using a LightCycler 480 (Roche Applied Science, Indianapolis, USA) (Kim et al., 2016). SYBR Green I Master Mix was used for mouse glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*), and the TaqMan probe (#04707494001, Roche Molecular Systems, Pleasanton, USA) method was used for 18S ribosomal RNA (*Rn18s*) as a housekeeping gene. The sequences of the primers used were as follows: forward 5′-AACCCATGCAAAGTCTACGG-3′ and reverse 5′-TGTGGAGAGAGGCAGGAACT-3′ for *G6pc*; forward 5′-AACCCATGCAAAGTCTACGG-3′ and reverse 5′-TGTGGAGAGAGGCAGGAACT-3′ for *Pck1*.

2.8. Hepatic glucose production in HepG2

Hepatic glucose production was determined in human hepatoma cells as reported elsewhere (Shannon et al., 2017). HepG2 (ATCC, HB-8065) cells were grown in Dulbecco's Modified Eagle's Medium