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Original article

Biological function of dipeptidyl peptidase-4 on type 2 diabetes patients and diabetic mice

Jing Qiao[a,*], Lei Li[a], Yanchun Ma[b], Ruhui Shi[c], Mei Teng[a]

[a] Department of VIP, Gaomi People's Hospital, Shandong, China
[b] Department of Ophthalmology, Gaomi People's Hospital, Shandong, China
[c] Department of Endocrinology, Gaomi People's Hospital, Shandong, China

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ABSTRACT

Background: Type 2 diabetes (TD2) is a sustained metabolic disorder, characterized by high blood glucose, insulin resistance (IR). Dipeptidyl peptidase-4 (DPP4) functions as an antigenic enzyme involved in hyperglycaemia, oxidative stress, and inflammation-associated IR. Therefore, association between DPP4 and TD2 warrants to be investigated.
Methods: In this study, blood samples of clinically diagnosed TD2 patients were harvested for biochemical tests. In addition, diabetic mice induced by high-fat diet (HFD) and single dose of streptozotocin (STZ) were used to assess the biological characteristics of DPP4 through biochemical and enzyme-linked immunosorbent assay (ELISA) tests, immunofluorescence staining, and western blot assay.
Results: Compared to controls, the clinical data of patients with TD2 resulted in increased contents of fasting blood glucose (FBG), glycated hemoglobin (HbA1c), homeostatic model assessment (HOMA)-IR, blood lipids of triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL-C), and interleukin 6 (IL6) in plasma samples ($p < 0.05$). Notably, blood levels of DPP4 in TD2 patients were increased significantly in comparison to that in non-diabetic adults ($p < 0.01$). In animal study, diabetic mice showed increased levels of glucose, insulin, lipids, DPP4 activity in sera. Visibly, hepatocellular DPP4 expression was up-regulated in diabetic mice. Interestingly, DPP4 inhibitor-treated mice showed significantly reduced DPP4 expression in serum ($p < 0.01$), and lowered DPP4-positive cells and protein content in the liver were observed when compared to those in diabetic mice ($p < 0.01$).
Conclusions: Collectively, these findings reveal that DPP4 biomolecule may be positively associated with TD2 development, and the underlying mechanism may be attributed to activation of DPP4 expression in liver cells.

1. Introduction

Diabetes mellitus (DM) refers to multiple metabolic symptoms maintained a prolonged period, in which the onset of TD2 accounts for about 90% of cases in DM [1]. TD2 patients may develop IR that a condition in cells fails to adequately respond to normal level of insulin. As the progresses of TD2, an insufficiency of insulin may also occur [2]. In the liver, insulin functionally limits release of glucose through gluconeogenesis. However, when the IR is formed, the liver cells induce glucose release into the blood [3]. In clinical treatment of TD2, there are several classes of anti-diabetic medications prescribed. In addition, exogenous injection of insulin can be used as monotherapy and or combined use with another oral medication [4]. However, health-associated adverse effects are still showed in existing medications, such as hypoglycemia found in insulin therapy. Thus, understanding of clinical characterization and health risks in TD2 may help prevent TD2 development and the complications. Dipeptidyl peptidase-4 (DPP4), also known as cluster of differentiation 26 (CD26), is a functional protein that is linked to signal transduction, immune regulation and cellular apoptosis. DPP4 can play a key role in glucose metabolism, in which it is responsible for the degradation of incretins, such as glucagon-like peptide-1 [5]. Some oral hypoglycemics, the specific DPP4 inhibitors, play effective therapeutic actions through suppressing the bioactivity of DPP4, thus induction of glycemia-lowering action [6]. Several studies indicate pathogenetic role of DPP4 in development of fibrosis in intracorporal organs, such as liver [7]. Therefore, we speculated that the enhanced DPP4 activity may be associated with TD2 development, in which DPP4-

* Corresponding author at: Department of VIP, Gaomi People's Hospital, No. 77, Zhenfu Street, Gaomi, Shandong, 261500, China.
E-mail address: jingqiao_666@126.com (J. Qiao).

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produced liver cells may be responsible for one of the underlying biological mechanisms. In this study, TD2 patients and HFD/STZ-diabetic mice were used to evaluate the biological function of DPP4 on metabolic functions, and to further discuss the proposed molecular mechanism.

2. Methods

2.1. Clinical study

Human cases, aged 40-60 years, were recruited from Department of Endocrinology of Gaomi People's Hospital (Shandong, China). A total of 50 patients were diagnosed as newly TD2 before treatment. The diagnostic standards of TD2 were referenced by the World Health Organization [8]. Meanwhile, additional non-TD2 adults (n = 50) were collected for comparable assays. In addition, these resultant TD2 cases and non-diabetic controls were further excluded to other diseases, such as enteritis, fatty liver, pancreatitis. This human study was approved by the Ethics Committee of Gaomi People's Hospital, and informed consents were obtained from subjects before conducting all experiments. In brief, blood samples were collected via anconal venous in heparinized vacuum tube, followed by preparation of plasma samples via 3000 g for 15 min at 4 °C. And then the plasma was tested at Clinical Laboratory for blood insulin, glycated hemoglobin, blood lipids, and inflammatory cytokines by using automatic analyzer (AU640, Olympus, Japan).

2.2. Animal study

Male Kunming mice, 6-week old, were purchased from the Qingdao Experimental Animal Center (No. SCXK20140001). This animal study was approved by the Ethical Committee of Weifang Medical University. The mice were housed in disinfectant cage under designed 20 ± 2 °C temperature and cycled light (12/12 h) each day.

As previously reported [9], the mice were fed with HFD (Slack Jingda Experimental Animal, Hunan, China) for 4 weeks, and then fresh-prepared STZ buffer (pH4.5, 0.1 mol/L in sodium citrate solution, 120 mg/kg) was intraperitoneally injected to mice. After 72 h, FBG was determined by using a glucose meter (Roche, USA). All mice with FBG greater than 11.1 mol/L were identified as TD2 mice [10]. The 20 TD2-diabetic mice were randomly assigned as 2 groups, TD2 group (n = 10); sitagliptin-treated group (Sita, a DPP4 inhibitor; 15 mg/kg per day; n = 10) for 7 days. Other 10 non-TD2 healthy mice were set as a control group. All mice were killed through cervical dislocation prior to blood collection at the end-point of this study. Serum samples were prepared and stored at −20 °C for further tests. In addition, liver samples were isolated for immunofluorescence stan and western blot assay.

2.3. Biochemical tests

The contents of TG, TC, LDL-C, high-density lipoprotein (HDL-C), alanine transaminase (ALT), aspartate transaminase (AST), and IL6, tumor necrosis factor alpha (TNFα), interferon gamma (IFN-γ), lactate dehydrogenase (LDH) was measured in sera (n = 10 in each group) by using a spectrophotometer (721 G, Keli, Shenzhen, China). Additionally, human and mouse DPP4 contents were determined by using commercially available ELISA kits (Cusabio Biotech, Wuhan, China) following by specifications.

2.4. Immunostaining protocols

Mouse liver tissues (n = 5 in each group) were fixed with 4% paraformaldehyde buffer and further prepared as 4 μm sections. The sections were dewaxed and post-blocked with 5% bovine serum albumin buffer for 2 hours at 37 °C. The sections were incubated with primary monoclonal antibody of mouse DPP4 (1:200, Santa Cruz, USA) at 4 °C overnight. After being washed with phosphate buffer saline for 3 times, sections were incubated with fluorescence-labeled goat anti-mouse IgG (Alexa Fluor 488, Beyotime, China), followed by DAPI dihydrochloride (Beyotime, China) to staining with nucleus. The final positive cells of DPP4 were calculated in at least 3 dissimilar views in each section under optical microscope [11–12].

2.5. Western blot protocols

Protein lysis of mouse liver sample (n = 3 in each group) was handled by using complete RIPA lysis buffer (Beyotime, China). And 30 μg protein lysis in each sample was separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by being transferred to polyvinylidene fluoride membrane (0.45 μm, Millipore, USA). After being blocked with 5% non-fat milk solution, the membrane was incubated with primary monoclonal antibody of mouse DPP4, beta-actin (1:1000, Santa Cruz, USA) at 4 °C overnight, followed by horse radish peroxidase (HRP)-coupled goat anti-mouse secondary antibody (1:5000, Beyotime, China). After being developed with BeyoECL Plus buffer (Beyotime, China), the membrane-based bands were imaged by using an Imager System (Bio-Rad, USA). The relative protein contents of DPP4 were calculated through normalization to beta-actin [13–14].

2.6. Statistical analysis

Statistical results were conducted by using statistical product and service solutions 19.0 software (Chicago, IL, USA). The final data are presented as mean ± standard deviation. And human data were assayed by Student t test when compared to controls. In addition, differences between the groups in mice were tested by using a one-way analysis of variance with a Tukey's test for post hoc two comparisons. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical comparable data of patients with TD2

In demographic data, these TD2 cases showed the average age of 49.5 ± 6.5 years, and the patient size of male and female was 36 and 14. Compared to healthy controls, the clinical data of TD2 patients showed increased levels of FBG, HbA1c, HOMA-IR, blood lipids of TG, TC, LDL-C, and IL6 in plasma samples ($p < 0.05$). In addition, blood contents of DPP4 in TD2 patients were elevated significantly in comparison to that in non-diabetic controls ($p < 0.01$) (Table 1).

3.2. Characteristics of metabolic parameters in TD2-diabetic and Sita-treated mice

To investigate DPP4-associated metabolic features in TD2, diabetic and DPP4 inhibitor-treated mice were used to further discussed. As revealed in Table 2, serological metabolic molecules of blood glucose, insulin, TG, TC, LDL-C, and liver functional enzymes of ALT, AST, LDH, as well as inflammatory cytokines of IL6, TNF-α in TD2-diabetic mice were significantly elevated when compared to those in control mice ($p < 0.05$). Interestingly, Sita-treated mice reversed these changes in TD2-diabetic mice with reductions of the biochemical parameters ($p < 0.05$). More notably, TD2-diabetic mice resulted in increased DDP4 contents in sera

Table 1
The clinical parameters of TD2 patients.

Characteristics	Controls	TD2
Age (years)	36.4 ± 7.8	49.5 ± 6.5
Sex (M/F)	20/15	36/14
BMI (kg/m^2)	21.5 ± 1.3	23.6 ± 2.8
FBG (mmol/L)	6.44 ± 0.86	15.67 ± 3.22[a]
Insulin (μU/mL)	7.62 ± 1.23	20.84 ± 2.41
HbA1c (mmol/mol)	30.62 ± 4.51	92.68 ± 7.82 [a]
HOMA-IR	3.2 ± 0.6	7.1 ± 1.8 [a]
TG (mmol/L)	1.61 ± 0.43	5.44 ± 1.20 [a]
TC (mmol/L)	2.03 ± 0.76	4.82 ± 1.03 [a]
LDL-C (mmol/L)	3.24 ± 1.81	6.74.1 ± 2.95 [a]
HDL-C (mmol/L)	3.67 ± 1.03	1.12 ± 0.53
IL-6 (pg/mL)	6.63 ± 2.51	10.35 ± 2.88 [a]
TNF-α (pg/mL)	18.92 ± 3.78	22.36 ± 5.64
DPP4 (nmol/mL)	16.19 ± 3.32	62.34 ± 2.38 [b]

Note:
M: male; F: female; BMI: body mass index; FBG: fasting blood glucose; HbA1c: glycated hemoglobin c; HOMA-IR: homeostatic model assessment of insulin resistance; TG: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha. When [a]$P < 0.05$ vs Controls; [b]$P < 0.01$ vs Controls.

Table 2
The metabolic parameters in TD2-diabetic and Sita-treated mice.

Parameters	Controls (n = 10)	TD2 diabetes (n = 10)	Sita treatment (n = 10)
FBG (mmol/mL)	4.26 ± 0.72	16.23 ± 2.16 [a]	8.21 ± 1.31 [b]
Insulin (mIU/L)	18.52 ± 2.26	43.59 ± 4.16 [a]	28.76 ± 2.35 [b]
TG (mmol/g)	1.32 ± 0.14	4.76 ± 0.52 [a]	2.66 ± 0.61 [b]
LDL-C (mmol/g)	1.89 ± 0.23	2.79 ± 0.82	2.06 ± 0.46
TC (mmol/g)	0.38 ± 0.06	1.45 ± 0.32 [a]	0.82 ± 0.09 [b]
HDL-C (mmol/g)	1.89 ± 0.14	0.56 ± 0.06 [a]	1.21 ± 0.48 [b]
ALT(U/L)	12.33 ± 2.04	32.42 ± 5.26 [a]	21.68 ± 3.15 [b]
AST(U/L)	20.26 ± 3.21	51.24 ± 6.03 [a]	36.27 ± 4.32 [b]
IL6(pg/mL)	15.07 ± 2.84	36.95 ± 4.58 [a]	28.42 ± 3.29
TNFα(pg/mL)	10.36 ± 1.29	25.46 ± 2.18 [a]	17.06 ± 1.36 [b]
IFN-γ(pg/mL)	24.56 ± 3.27	30.72 ± 4.24	32.56 ± 2.87
LDH(U/L)	134.58 ± 16.26	268.65 ± 23.45 [a]	187.61 ± 10.62 [b]
DPP4(pg/mL)	30.64 ± 6.37	162.84 ± 18.42 [c]	42.71 ± 2.33 [d]

Abbreviation:FBG, fasting blood glucose; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, glutamic-pyruvic transaminase; AST, glutamic-oxaloacetic transaminase; IL6, interleukin 6; TNFα, tumor necrosis factor alpha; IFN-γ, interferon gamma; LDH, lactate dehydrogenase. When [a]$p < 0.05$ vs Controls, [c]$p < 0.01$ vs Controls; [b]$p < 0.05$ vs TD2-diabetes, [d]$p < 0.01$ vs TD2-diabetes.

when compared to diabetes-free mice ($p < 0.01$). In addition, reduced DDP4 levels were observed in Sita-treated mice when compared to TD2-diabetic mice ($p < 0.01$).

3.3. Biological characteristics of DPP4 on TD2-diabetic and Sita-treated mice

In order to further characterize immunophenotype of DPP4 in liver cells, immunofluorescence staining was conducted. As shown in Fig. 1, TD2-diabetic mice resulted in increased hepatocellular TUNEL-positive and DPP4-labeled cells, in which the cell counts were greater than those in controls ($p < 0.01$). Interestingly, Sita-treated mice showed decreased those positive cells of TUNEL-positive and DPP4-labeled cells in the livers of TD2-diabetic mice ($p < 0.01$). In data validation, western blot results indicated that TD2-diabetic mice had elevated expression of DPP4 in liver cells ($p < 0.01$). However, intrahepatic down-regulation of DPP4 level was observed in Sita-treated mice ($p < 0.01$).

4. Discussion

In human data, pathogenic factors of TD2 were linked to high blood glucose and lipids, insulin resistance, and inflammatory stress, as revealed in Table 1. Additionally, positive association between DPP4 bioactivity and TD2 condition was found, characterized with significant elevation of blood DPP4. These clinical

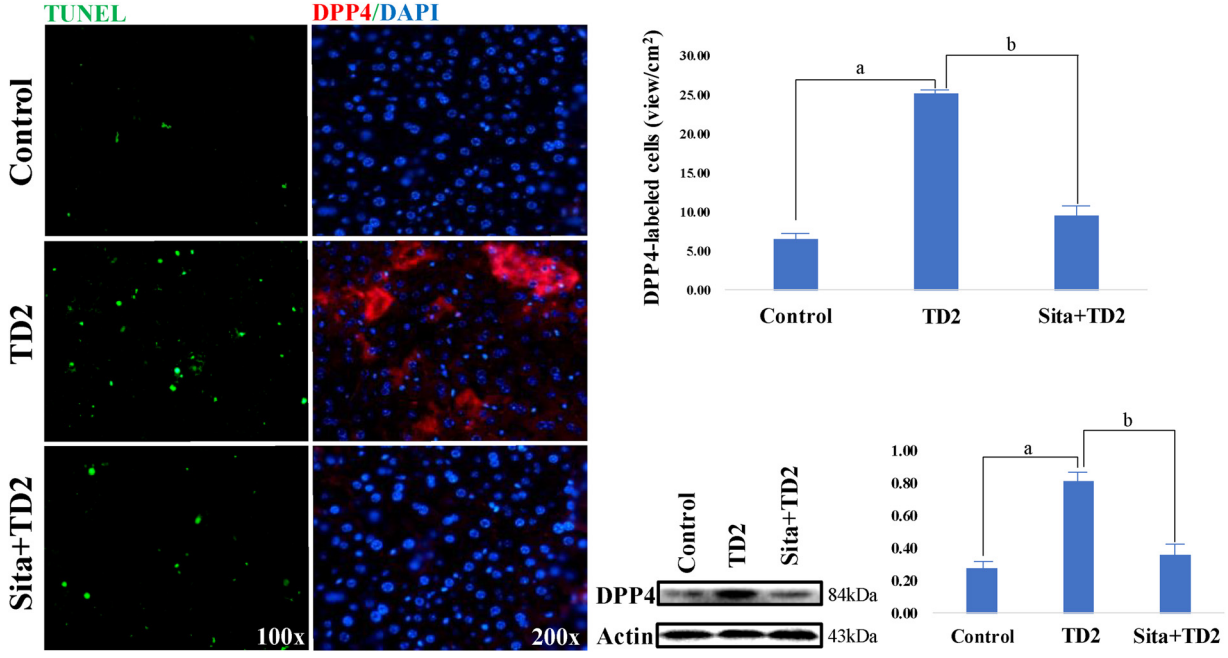

Fig. 1. Characterization of DPP4 on TD2-diabetic and Sita-treated mice.
As results, TD2-diabetic mice showed increased TUNEL-positive and DPP4-labeled cell counts in liver cells, as revealed in immunofluorescence stains (100× and 200×, magnification). However, Sita-treated mice resulted in reduced positive cells of hepatic TUNEL and DPP4. Further, western blot data indicated that TD2-diabetic mice caused elevated DPP4 protein expression in the liver, while down-regulation of hepatocellular DPP4 protein level was observed in Sita-treated mice. When [a]$p < 0.01$ vs. control; [b]$p < 0.01$ vs. TD2.

findings highlighted that DPP4 might serve as a potential diabetogenic molecule in TD2 development. However, the underlying mechanism behind this biological action needs to be investigated.

In physiological function, DPP4 plays an important role in glucose metabolism [15]. Previous studies show biological role of DPP4 on hyperglycaemia, positive association between blood DPP4 and HbA1c in TD2 [16]. In addition, sustained high blood glucose can cause significant elevation of plasma DPP4 levels in TD2 patients [17]. On the basis of current human data, we reasoned that marked increment in blood DPP4 content at early stage of TD2 might indicate impaired glucose homeostasis, as well as induce development of hyperglycaemia and insulin resistance.

The HFD-induced TD2 animal model aims to investigate diabetic topic, and it is designed to mimic the similar metabolic disorders of TD2 in humans, commonly using mice and rats [18]. In addition, STZ is previously used an antineoplastic drug that is special toxic to pancreatic beta cells in mammals. Therefore, small dose of STZ may effectively increase the success of TD2 when exposure to HFD [19]. Mice are typically used by scientists for establishment of TD2 model in experiments because the rodents have mammalian metabolic systems similar to those in human [20]. In this study, TD2-diabetic mice were established for assessing biological expressions of DPP4 in blood and liver cells. As results, significant elevation of DPP4 contents in blood and liver cells were found in TD2-diabetic mice. In addition, TUNEL-positive cells were elevated in liver cells of TD2-diabetic mice. Therefore, these findings highlighted that increased hepatic cell death induced by HFD and STZ might notably leads to a further increment in DPP4 expression. In addition, hepatocellular elevated DPP4 protein levels might induce possible adverse outcomes of hyperglycaemia and insulin resistance. Therefore, inhibition of hepatic DPP4 activity seems to be a promising strategy to control TD2.

DPP4-based hypoglycemics are a group of zymological inhibitors that suppress the activity of DPP4, and these medicines are widely used for therapy of TD2 [21]. Blocking DPP4 production can enhance the activity of incretin that exerts key role in insulin release and blood glucose regulation [22]. Sitagliptin (Sita) is an oral antihyperglycemic drug for clinical treatment of TD2 patients [23]. In preliminary mechanism, Sita plays the therapeutic effectiveness through suppression of biological activity of DPP-4 [24]. In this study, Sita-treated TD2-diabetic mice resulted in reduced blood glucose, blood lipids, and inflammatory cytokines. Furthermore, DPP4 contents in blood and liver were lowered, accompanied with reduced cell death in the liver. Therefore, direct suppression of DPP4 activity in liver cells may alleviate the pathological development of TD2.

5. Conclusion

Based on the current data of human and mice, we initially conclude that elevated blood of DPP4 bioactivity may be positively associated with TD2 development. In addition, the underlying molecular mechanism behind this action may be linked to induction of DPP4 expression in liver cells.

Declaration of interest

The authors declare that they have no competing interests.

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