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General Medicine

LINAGLIPTIN MODIFIES PLATELET ACTIVATION AND ARTERIAL THROMBOSIS BY REGULATING MITOCHONDRIAL RESPIRATORY RESERVE

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KEYWORDS :

INTRODUCTION

DPP (dipeptidyl peptidases), a family of intracellular or extracellular exopeptidases that cleave X-proline dipeptides from the polypeptide N terminus, cleaves the incretin hormone GLP-1 (glucagon-like peptide 1) and thereby regulates glucose metabolism, [1] this enzyme has been highly investigated in the context of type 2 diabetes mellitus. Enhanced secretion of GLP-1 on food uptake activates the GLP-1R (GLP-1 receptor) on pancreatic β cells, triggering insulin release and subsequently reducing blood glucose level. DPP-4 rapidly cleaves the native intact GLP-1 forms, that is, GLP-1(7-36) amide and GLP-1(7-37), into GLP-1(9-36) amide and GLP-1(9-37), respectively, both of which are incapable of stimulating GLP-1R.[2] Accordingly, inhibitors of DPP-4, such as sitagliptin and linagliptin, markedly prolong the half-life of native GLP-1 and are being successfully applied to reduce hyperglycaemia in patients with diabetes mellitus.[1,3]

Diabetes mellitus patients have a 2- to 4-fold increased risk of cardiovascular disease and a mortality rate comparable to myocardial infarction patients[4,5]. Platelet reactivity is a strong independent predictor of major adverse cardiac events in diabetes mellitus[5,6]. Diabetes mellitus is linked to increased atherosclerosis and thrombosis, which are underlying pathologies of cardiovascular disease. 7–9 Antiplatelet therapy has been shown to reduce vascular events in patients with diabetes mellitus who do not have cardiovascular disease[11]. First indications that DPP-4 activity can affect platelet activation came from a study reporting that platelets from patients with diabetes mellitus treated with the DPP-4 inhibitor sitagliptin displayed reduced aggregation in vitro.[12] A later study showed increased platelet aggregation in Glp1r-deficient mice, along with increased cAMP-mediated signalling in platelets stimulated with the GLP-1R agonist exendin-4.[13] Furthermore, the GLP-1R agonist exenatide (with sequence similarity to exendin-4) was found to suppress aggregation responses of human and mouse platelets, paralleled by cAMP changes.[14] Together, these observations supported the concept that the DPP-4-GLP-1R axis can regulate platelet functions.

Linagliptin, is a competitive inhibitor of DPP-4 in various tissues.[15,16,17] In rodents, linagliptin was seen to be more effective than sitagliptin at reducing DPP-4 activity in tissue and related inflammatory properties.[18,19] The significance of platelet homeostasis was emphasized by the recent discovery of platelet mitochondrial dysfunction in type 2 diabetes.[20,21]

Deficiency or Inhibition of DPP-4 in Mice Suppresses Thrombus Formation Under Flow Without a Main Role for Platelet GLP-1R

To study the effect of DPP-4 on platelet responses, it was studied that thrombus formation in perfused whole blood from Dpp4−/− mice in comparison to corresponding wild-type mice. In whole blood samples perfused over collagen I at arterial wall-shear rate ex vivo, platelet activation and characteristics of thrombus formation were measured by multicolour microscopy (Figure 1 A). Based on the microscopy images, a subtraction heatmap comprising of scaled values of 8 parameters of thrombus formation was generated and filtered for large effect sizes (Cohen's d ≥0.8). This showed a reduction in thrombus formation on DPP-4 deficiency, as apparent from the observed reductions for thrombus morphology (4.51±0.41 versus 3.71±0.63) and contraction scores (2.32±0.56 versus 1.98±0.70; Figure 1 B). Along the same line, treating wild-type mice for 5 days with the DPP-4 inhibitors linagliptin and sitagliptin, reduced platelet responses under flow, as demonstrated by reductions in thrombus morphology, contraction, and multilayer scores, as well as by reduced platelet deposition (quantified as platelet SAC and platelet multilayer SAC; Figure 1 C).[22]

Platelet counts were not affected by DPP-4 deficiency or inhibition, and neither were white nor red blood cell counts. Treating Dpp4−/− mice with linagliptin or sitagliptin could not further reduce responses of platelets under flow (Figure 1C), indicating that the inhibitory effects of linagliptin and sitagliptin on the thrombotic process are mediated by interference with DPP-4. [22]

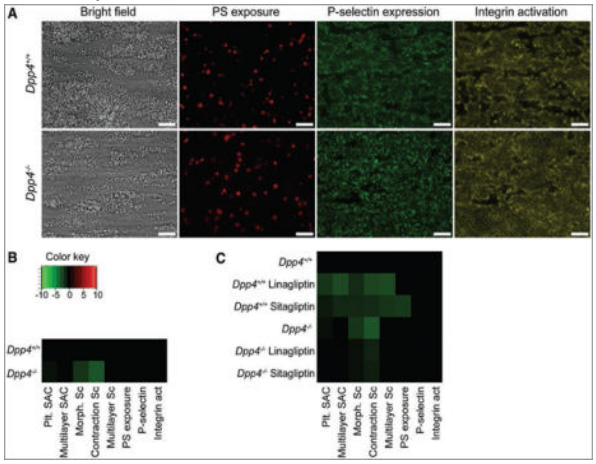

Figure 1. Murine DPP-4 (dipeptidyl peptidase 4) deficiency impairs thrombus formation under flow.

Blood from Dpp4−/− or wild-type control mice was perfused over collagen I microspots at 1000/s. Where indicated, mice were fed for 5 d a normal laboratory diet supplemented with linagliptin or sitagliptin. Thrombi formed were poststained with fluorescein isothiocyanate (FITC)- labelled anti-P-selectin mAb (secretion), PE-labelled JON/A mAb (integrin activation) and AF647-annexin A5 (phosphatidylserine [PS] exposure). A, Representative brightfield and fluorescence images. Scale bar =20 μm. B, Subtraction heatmap of scaled values,

compared to Dpp4+/+, filtered for differences with large effect size (Cohen's d ≥0.8); n=23–26. C, Subtraction heatmap of scaled values, compared to wild type without treatment, filtered for differences with large effect size (Cohen's d ≥0.8); n=6–13. SAC indicates surface area coverage.

Linagliptin Regulates the Mitochondrial Respiratory Reserve to Alter Platelet Activation and Arterial Thrombosis

A study was carried out to test the hypothesis that inhibition of DPP-4 can inhibit platelet activation and arterial thrombosis by preventing platelet mitochondrial dysfunction and release. The effects of pharmacological DPP-4 inhibition on carotid artery thrombosis, platelet aggregation, and platelet mitochondrial respiration signalling pathways were studied in mice[23]

Linagliptin Inhibits Arterial Thrombosis After Vascular Injury

Using the well-established common carotid artery injury thrombosis model, it was determined that compared with the control PBS, linagliptin inhibited thrombus growth in adult male mice. The average occlusion time was significantly prolonged in linagliptin-treated mice (479 ± 24.1 s, n = 6) compared with the vehicle mice (163.7 ± 38.2 s, n = 6, p < 0.001 vs. linagliptin group) after the initiation of arterial injury (Figures 2A, B). Therefore, linagliptin has an inhibitory effect on the artery thrombosis model, but it is only effective at a high dose (20 mg/kg/day) in mice.[23]

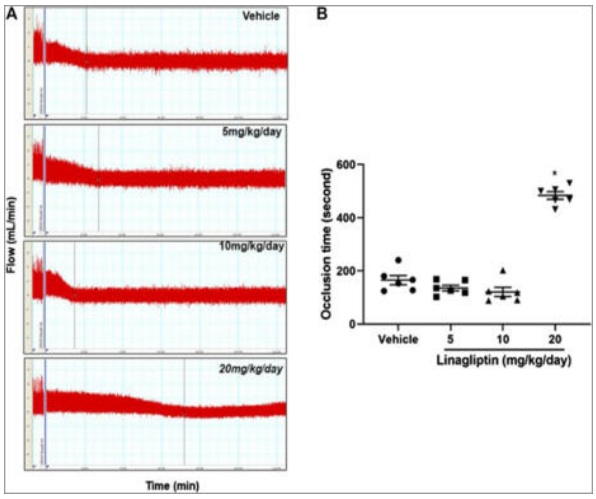

Figure 2: Linagliptin regulates thrombus formation after carotid artery injury in mice fed normal chow.

(A) Linagliptin or vehicle control was administered by gavage (5, 10, or 20 mg/kg/day; once daily for 7 days) to WT mice before injuring carotid arteries with topical FeCl3. Carotid blood flow tracings are shown. (B) Mean carotid artery occlusion times after FeCl3 injury (n = 6/group); All data were expressed as the means ± SEM. *p < 0.05 vs. 5 and 10 mg/kg/day and vehicle control groups.

Linagliptin Inhibits Thrombin-Induced Platelet Adhesion and Aggregation In Vitro

Pre-treatment with linagliptin inhibited thrombin-induced platelet aggregation. As shown in Figures 3A–D, the results were expressed as % inhibition in a representative aggregation assay. Linagliptin demonstrated dose-dependent inhibition of thrombin (0.1 U/ml)-induced platelet aggregation (Figures 3C, D), while linagliptin exhibited only a mild inhibitory effect thrombin (0.05 U/ml)-induced platelet aggregation at a concentration of 10 μM (Figures 3A, B). Similarly, high dose of linagliptin inhibited collagen but not ADP-induced platelet aggregation (Figures 3E–H). Moreover, pre-treatment with linagliptin (100 μM) inhibited thrombin-induced platelet adhesion (Figures 3I, J). To examine the significance of the findings in a diabetic model, the study group fed mice high-fat chow (HFD) for 14 weeks, which produced obesity, hyperglycaemia. Thrombin (0.1 U)-stimulated platelet aggregation was significantly increased in HFD/STZ mice compared with normal diet (ND) mice (Figures 3C, D) (71.6 ± 5.5% vs. 53.8 ± 2.02%; p < 0.05). The high dose of linagliptin (100 mM) also inhibited thrombin-stimulated platelet aggregation in HFD mice compared to ND mice. These findings showed that diabetic platelets were more sensitive to thrombin-induced aggregation than non-diabetic platelets, and that non-diabetic platelets were more susceptible to linagliptin-induced aggregation[23]

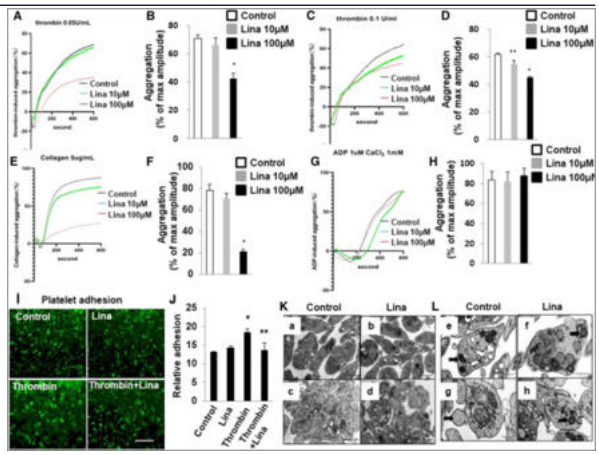

Figure 3: Treatment with linagliptin attenuates thrombin-induced platelet aggregation in vitro.

Representative aggregometry traces of mouse platelets incubated for 10 min with linagliptin (10–100 μM) and stimulated to aggregate with 0.05 U/ml (A,B) or 0.1 U/ml (C,D) of thrombin. (B, D): Error bars represent the SEM. n = 4–7/group. *p < 0.05 vs. vehicle and linagliptin (10 μM); **p < 0.05 vs. vehicle. E–H: Representative aggregometry traces of mouse platelets incubated for 10 min with linagliptin (10–100 μM) and stimulated to aggregate with 5 μg/ml collagen (E, F) and 10 μM ADP (H, I). *p < 0.05 vs. vehicle and linagliptin (10 μM); (I, J): Effects of Lina on mice platelet adhesion. (I) Representative of images of platelet adhesion after Lina treatment, platelets were pre-labelled with CMFDA, scale bar = 20 μm. (J) Quantification of the relative adhesion by pixel density measurements in platelets. All data were expressed as the means ± SEM. Statistical comparisons were performed using the two-way ANOVA. *p < 0.05 vs. vehicle and linagliptin (10 μM). (K, L): Platelets were prepared for transmission electron microscopy imaging, and images were acquired using a Tecnai-12 electron microscope at ×4,300 magnification. (K) The distribution of granules (L) (scale bar: 1 μm) and mitochondria (F) (dark arrow) (scale bar: 500 nm) throughout the platelet. Images are representative of three independent observations. Resting: a, b, e, f; Thrombin: c,d,g,h.

Effect of Linagliptin on In Vivo Mitochondrial Respiration in Treated Mouse Platelets

Platelets from mice treated with linagliptin showed similar mitochondrial changes to the in vitro findings, characterized by reduced OCR during activation (Figure 5).[23]

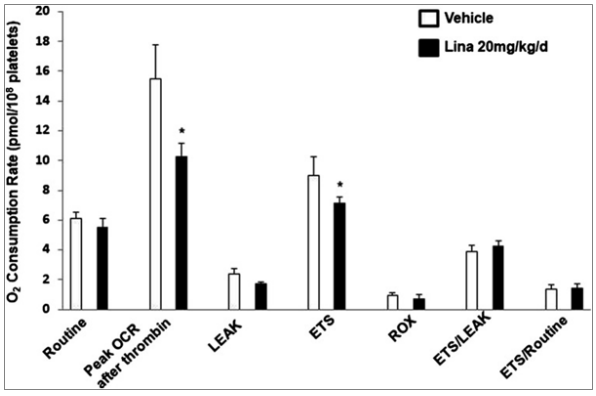

Figure 5: Effect of linagliptin on in vivo mitochondrial respiration in treated mouse platelets.

Platelets were obtained from mice after 7 days of treatment with linagliptin or vehicle. Platelets were assayed for oxygen consumption in an Oxygraph-2k high resolution respirometer. All data were expressed as the means ± SEM. n = 3/each group. Statistical comparisons were performed using the paired Student's t-test.

Data from graph are normally distributed according to Kolmogorov-Smirnov normality test. *p < 0.05 vs. vehicle. ETS, maximal electron transfer system capacity; ROX, residual oxygen concentration; LEAK, leak-state respiration (non-ADP-stimulated respiration); OXPHOS, oxidative phosphorylation capacity (ADP-stimulated

respiration).

Linagliptin Preserves the Activation of Cyclic AMP-Phosphodiesterases Induced by Thrombin

DPP-4 activity in the lysates of platelets treated with thrombin was significantly higher than that in the lysates of the control group (Figure 6 A). Consistent with this finding, thrombin or vehicle control was added for 10 minutes, after which platelet lysates were prepared, and assessed by Westernblot analysis. Thrombin stimulated a significant increase in DPP-4 expression in platelets (Figure 6 B), suggesting a significant role for DPP-4 in the activation of platelets.[23]

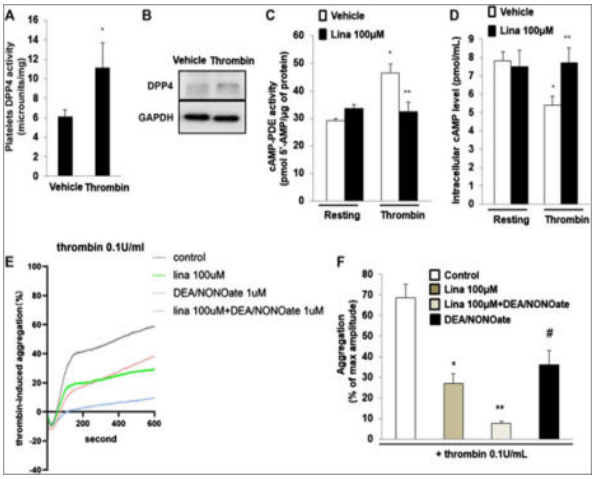

Figure 6: Linagliptin preserves the activation of cyclic AMP (cAMP)-phosphodiesterases (PDEs) induced by thrombin.
(A). dipeptidyl peptidase-4 (DPP-4) activity was measured in lysates of platelets treated with thrombin (0.1 U/ml for 10 min) or vehicle. Data are the mean of triplicate experiments and are presented as the mean ± SEM. Data from graph are normally distributed according to Kolmogorov-Smirnov normality test. (B). Expression of DPP-4 in in lysates of platelets was evaluated by western blot analyses. GAPDH was used as an internal control. Image is representative of three independent experiments. PDE activity (C) and intracellular cAMP level (D) measured in lysates of platelets treated with thrombin (0.1 U/ml for 10 min) in the presence or absence of linagliptin (100 μM). Data are presented as the mean ± SEM. Statistical analysis: one-way ANOVA with Bonferroni's multiple comparisons test. *p < 0.05 vs. vehicle; **p < 0.05 vs. thrombin alone. n = 3–6/group. (E, F). Linagliptin increases the inhibition of platelet aggregation by nitric oxide (NO). Mouse platelets were pre-treated with 1 mM DEA-NONOate for 15 min.

Representative aggregometry traces of platelets incubated for 10 min with linagliptin (100 μM) and stimulated to aggregate with 0.1 U/ml of thrombin. All data were expressed as the means ± SEM. n = 3–4/group. All data were expressed as the means ± SEM. Statistical comparisons were performed using ANOVA. *p < 0.05 vs. thrombin alone; **p < 0.05 vs. linagliptin; #p < 0.05 vs. thrombin alone and linagliptin.

PDE activity was examined in platelet lysates by using a cAMP-PDE enzymatic activity assay. The results showed that thrombin induced PDE activity, while pre-treatment with linagliptin preserved PDE activity (Figure 6C).[23]

SUMMARY

DPP-4, a widely expressed cell surface peptidase, activates intracellular signal transduction pathways via the cell membrane. Linagliptin is a DPP-4 inhibitor used to treat type 2 diabetes. Like sitagliptin and saxagliptin, linagliptin slows the breakdown of endogenous incretin hormones like GLP-1 and GIP. However, the drug's cardiovascular safety has not been established. Antagonizing thrombin-induced platelet activation, Linagliptin reduces markers of arterial thrombosis formation independent of glucose control. Most agonist-induced platelet cAMP production was linked to mitochondrial function and aggregation. By decreasing PDE activation, Linagliptin increased cAMP-mediated signalling. Due to cAMP-mediated signalling, linagliptin may suppress thrombin-induced mitochondrial PDE responses and thus platelet aggregation. Alternatively, DPP-4 blockade and NO were linked to platelet activation (Figure 7)[23]

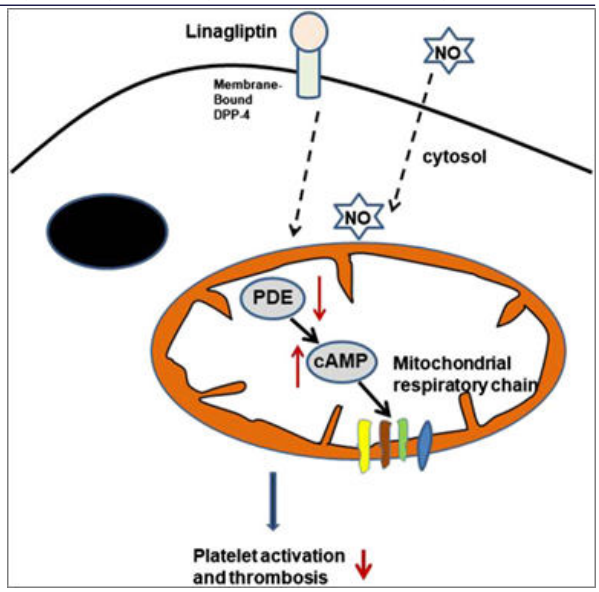

Figure 7: Potential GLP-1-independent mechanism of DPP-4 inhibition by linagliptin in the regulation of platelet activation.
cAMP is formed inside the mitochondrial matrix and stimulates the respiratory chain. The cAMP signals are terminated through degradation by PDE in the presence of linagliptin. cAMP, cyclic AMP; PDEs, phosphodiesterases; NO, nitric oxide.

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