Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Native, Intact Glucagon-Like Peptide 1 Is a Natural Suppressor of Thrombus Growth Under Physiological Flow Conditions

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OBJECTIVE: In patients with diabetes mellitus, increased platelet reactivity predicts cardiac events. Limited evidence suggests that DPP-4 (dipeptidyl peptidase 4) influences platelets via GLP-1 (glucagon-like peptide 1)-dependent effects. Because DPP-4 inhibitors are frequently used in diabetes mellitus to improve the GLP-1-regulated glucose metabolism, we characterized the role of DPP-4 inhibition and of native intact versus DPP-4-cleaved GLP-1 on flow-dependent thrombus formation in mouse and human blood.

APPROACH AND RESULTS: An ex vivo whole blood microfluidics model was applied to approach in vivo thrombosis and study collagen-dependent platelet adhesion, activation, and thrombus formation under shear-flow conditions by multiparameter analyses. In mice, in vivo inhibition or genetic deficiency of DPP-4 (*Dpp4*$^{-/-}$), but not of GLP-1-receptors (*Glp1r*$^{-/-}$), suppressed flow-dependent platelet aggregation. In human blood, GLP-1(7-36), but not DPP-4-cleaved GLP-1(9-36), reduced thrombus volume by 32% and impaired whole blood thrombus formation at both low/venous and high/arterial wall-shear rates. These effects were enforced upon ADP costimulation and occurred independently of plasma factors and leukocytes. Human platelets did not contain detectable levels of GLP-1-receptor transcripts. Also, GLP-1(7-36) did not inhibit collagen-induced aggregation under conditions of stirring or stasis of platelets, pointing to a marked flow-dependent role.

CONCLUSIONS: Native, intact GLP-1 is a natural suppressor of thrombus growth under physiological flow conditions, with DPP-4 inhibition and increased intact GLP-1 suppressing platelet aggregation under flow without a main relevance of GLP-1-receptor on platelets.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: diabetes mellitus ■ dipeptidyl peptidase 4 ■ glucagon-like peptide 1 ■ glucose ■ platelets

DPP (dipeptidyl peptidases) consists of a family of intracellular or extracellular exopeptidases that cleave X-proline dipeptides from the polypeptide N terminus. Since DPP-4 (dipeptidyl peptidase 4 or CD26) 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 consequently 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 linagliptin and sitagliptin, markedly prolong the half-life of native GLP-1 and are being successfully applied to reduce hyperglycemia in patients with diabetes mellitus.[1,3]

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Nonstandard Abbreviations and Acronyms

CRP-XL	collagen-related peptide
DPP-4	dipeptidyl peptidase 4
FITC	fluorescein isothiocyanate
GLP-1	glucagon-like peptide 1
GLP-1R	glucagon-like peptide 1 receptor
PRP	platelet-rich plasma
SAC	surface area coverage
VWF	von Willebrand factor

Patients with diabetes mellitus have a 2- to 4-fold higher risk to develop cardiovascular diseases and, at least in some studies, encounter a mortality rate as high as patients with a previous myocardial infarction.[4,5] In diabetes mellitus, increased platelet reactivity was found to be a strong independent predictor of major adverse cardiac events.[5,6] In line with platelet hyperactivity, diabetes mellitus is also associated with increased atherosclerosis and thrombosis as underlying pathologies of cardiovascular disease.[7–9] Antiplatelet therapy in patients with diabetes mellitus has proven to be beneficial for secondary prevention of atherothrombotic recurrences[10] and can also reduce vascular events in the absence of cardiovascular disease.[11]

First indications that DPP-4 activity can affect platelet activation came from a study revealing 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 signaling 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. However, mechanistic insights how DPP-4 inhibition affects platelet properties in the context of thrombus formation are still missing. Furthermore, albeit previous studies examined synthetic GLP-1R agonists (exendin-4 and exenatide, with only ≈50% sequence identity to native GLP-1[15]), the regulation of platelet responses by native, uncleaved GLP-1 has not been investigated. Considering that ex vivo measurements of collagen-dependent thrombus formation under flow can be used as a validated proxy measure of arterial thrombosis in vivo,[16] the present study used a whole blood microfluidics assay in a multiparameter setting[17] for detailed assessment of alterations in thrombus formation triggered by (1) DPP-4 inhibition and (2) native uncleaved versus DPP-4-cleaved GLP-1 forms.

Highlights

- Inhibition of DPP-4 (dipeptidyl peptidase 4) enzyme activity in vivo, genetic deficiency of DPP-4 and increased levels of native GLP-1(glucagon-like peptide 1[7-36]) suppress collagen-mediated thrombus formation under physiological flow conditions.
- Suppression of platelet responsiveness by native GLP-1(7-36) requires flow conditions but occurs independently of plasma factors and without a main relevance of GLP-1R on platelets.
- These findings contribute a mechanistic explanation for the cardiovascular benefit of true GLP-1(7-36)–mimetics but not of exendin-based GLP-1R agonists.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and the online-only Data Supplement files. A detailed version of Materials and Methods is available in the online-only Data Supplement.

Major Resources

Please see the Major Resources Table in the online-only Data Supplement.

Human Blood and Platelet Isolation

Experiments were approved by the local Medical Ethics Committees (EK191/14, University Hospital Aachen; and METC 10-3-023, Maastricht University). Human blood was drawn from healthy volunteers after full informed consent, conform the Declaration of Helsinki. Blood was collected into 3.2% trisodium citrate; the first 3 mL were discarded. Platelet-rich plasma (PRP) was obtained by centrifuging citrate anticoagulated blood at 250*g* for 15 minutes, and platelet-free plasma was collected by centrifuging twice at 2150*g* for 10 minutes. To obtain washed platelets, PRP was centrifuged at 2200*g* for 2 minutes, the remaining pellet was washed with HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer (10 mmol/L HEPES, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mg/mL glucose, 1 mg/mL bovine serum albumin, pH 6.6), before washed platelets were suspended into HEPES buffer pH 7.45. Platelet count was determined using a Sysmex XN-350 hematology analyzer (Sysmex, Chuo-ku Kobe, Japan).

Mouse Blood for Platelet Studies

All animal experiments were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany, approval number 81-02.04.2017.A413; and by the Landesuntersuchungsamt Koblenz, approval numbers G17-1-050 and G14-1-039) and complied with the German animal protection law. To study platelet activation, blood was obtained from mice with deficiencies in *Glp1r* or *Dpp4*,[18,19] both on a C57BL/6J genetic background. DPP-4 knockout mice (*Dpp4*$^{-/-}$) were bred in Mainz or Aachen; they were originally a kind gift of Dirk Reinhold (Otto-von-Guericke-University Magdeburg, Magdeburg, Germany) and generated by Didier Marguet (Centre

d'Immunologie de Marseille-Luminy, Marseille, France).[18] GLP-1R knockout mice (*Glp1r*$^{-/-}$) were bred in Mainz; they were originally obtained from Charles River (Sulzfeld, Germany) and generated by Daniel Drucker (Mt Sinai Hospital, Toronto, Canada).[19] Wild-type control mice of the same background came from the same breeding program. Because initial characterization of DPP-4-knockout animals did not reveal differences between sexes,[18] we have conducted our experiments in mixed-sex, using age- and sex-matched littermates between experimental groups. All experiments were performed using age- and sex-matched littermates. Where indicated, *Dpp4*$^{-/-}$ mice were fed a normal laboratory diet (Sniff PS R/M-H mod.) supplemented with linagliptin (83 mg/kg diet) or sitagliptin (280 mg/kg diet) for 5 days. The mice were anesthetized by an intraperitoneal injection of 100 mg/kg per body weight ketamine and 10 mg/kg xylazine. Blood from mice was collected into 40 µmol/L PPACK, 5 U/mL heparin and 40 U/mL fragmin or trisodium citrate.

DPP Activity Measurements

DPP-4 activity was measured in lysates and releasates from stimulated human platelets. Therefore, washed platelets (1×10^7/mL) were stimulated with thrombin (1 U/mL), ionomycin (10 µmol/L), or CRP-XL (collagen-related peptide; 5 µg/mL) in the presence of 2 mmol/L $CaCl_2$ for 45 minutes at 37°C. As a negative control, unstimulated platelets were treated with prostacyclin (0.1 µg/mL) in the presence of 2 mmol/L $CaCl_2$. In all cases, platelet releasates and lysates of pelleted platelets were analyzed. The pelleted platelets (1×10^6) were lysed with 360 µL cell lysis buffer, as described.[20] DPP activity was measured using the DPP-IV-Glo protease assay (Promega) according to the manufacturer's instructions; per test 25 µL releasate or lysate was used plus 25 µL assay reagent. The activity of DPP-4 plus that of its closest family members DPP-8 and DPP-9 (containing similar cleavage recognition motif and pH optimum) contribute to the luminescent read out in this assay; this is referred to as DPP-4-like activity. Specific quantification of DPP-4 or DPP-8/9 activities was performed by addition of the DPP-8/9 inhibitor 1G244 or the DPP-4 inhibitor sitagliptin, respectively, at final concentrations of 7 µmol/L in the assay (30 minutes treatments). Measurements gave total DPP-4-like activity as well as specific DPP-4 and DPP-8/9 activity in platelets and platelet-free plasma.[21]

Whole Blood Thrombus Formation

An established whole blood microfluidics assay in a multiparameter setting was applied for a detailed assessment of alterations in thrombus formation under physiological flow conditions, as described.[17] Glass coverslips were coated with 2 µL microspots of collagen type I (100 µg/mL) and collagen type III (100 µg/mL) and were subsequently blocked with 1% bovine serum albumin. Citrated mouse blood was recalcified (3.75 mmol/L $MgCl_2$, 7.5 mmol/L $CaCl_2$) in the presence of PPACK (40 µmol/L) and subsequently, perfused over the coated surfaces for 3.5 minutes at a wall-shear rate of 1000/s. Mouse blood collected into PPACK anticoagulant medium did not require recalcification. Where indicated, blood samples were pretreated with iloprost (0.55 µmol/L) or exendin-9 (10 nmol/L) for 30 minutes; or alternatively with sitagliptin (7 µmol/L) in combination with GLP-1(7-36) (50 nmol/L) or vehicle for 10 minutes before perfusion. Thrombi formed after 3.5 minutes of flow were directly postlabeled with a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin mAb (1:40), PE-labeled JON/A mAb (1:20; to analyze integrin activation) and AF647-annexin A5 (1:200; to analyze exposure of phosphatidylserine) in modified Tyrode's buffer (136 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH_2PO_4, 5 mmol/L HEPES, 2 mmol/L $MgCl_2$, 1 mg/mL glucose, and 1 mg/mL bovine serum albumin, pH 7.45) containing $CaCl_2$ (2 mmol/L) and heparin (1 U/mL).

For formation of human platelet thrombi, citrated blood from healthy subjects was recalcified (3.75 mmol/L $MgCl_2$; 7.5 mmol/L $CaCl_2$) in the presence of PPACK (40 µmol/L) and perfused over collagen I surface at given wall-shear rates (6 minutes at 150/s; 3.5 minutes at 1000/s or 1700/s).[22] Where indicated, blood samples were preincubated with sitagliptin for 10 minutes (7 µmol/L) plus GLP-1(7-36) (50 nmol/L), GLP-1(9-36) (50 nmol/L) or vehicle, and coperfused with 2-methylthioadenosine diphosphate (1 µmol/L, f.c.). Human thrombi formed on the surfaces were stained with AF647-conjugated anti-P-selectin mAb (2 µg/mL), FITC-labeled anti-fibrinogen mAb (1:100; to quantify fibrinogen binding as read out for integrin activation), and AF568-annexin A5 (1:200) in HEPES buffer (10 mmol/L HEPES, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mg/mL glucose, and 1 mg/mL bovine serum albumin) containing $CaCl_2$ (2 mmol/L) and heparin (1 U/mL).

Per flow run, series of representative brightfield and fluorescence images were captured for analysis of surface area coverage (SAC) by adhered platelets (platelet SAC) as well as for analysis of platelet activation markers (phosphatidylserine exposure, P-selectin expression, integrin activation) using scripts written in Fiji software, as described.[17,23] Furthermore, based on the brightfield images of adhered platelets, effects on platelet aggregation and thrombus formation were assessed by quantifying the multilayer SAC as well as by determining a morphological score, contraction score, and multilayer score. These scores were determined by visual inspection of the platelet features per microspot in comparison to preset reference images, using the following criteria[17]: morphological score (range, 0–5): 0, no or hardly any adhered platelets; 1, multiple single-adhered platelets; 2, extensive coverage of single-adhered platelets; 3, small platelet aggregates; 4, intermediate platelet aggregates; and 5, thrombi with large-size platelet aggregates. Contraction score (range, 0–3): 0, no contraction; 3, close contraction. Multilayer score (range, 0–3): 0, no platelet layers; 3, multilayered aggregates.[24] Observers were blinded to the experimental condition.

For quantification of thrombus volume and height, blood samples were incubated with sitagliptin (7 µmol/L) in combination with vehicle, GLP-1(7-36) (50 nmol/L) or GLP-1(9-36) (50 nmol/L) for 10 minutes at room temperature. The membrane probe $DiOC_6$ (0.5 µg/mL) was added to the blood samples before recalcification to label the platelets. After blood perfusion for 3.5 minutes at 1000/s, z-stacks of confocal fluorescence images were recorded with a spacing of 0.5 µm using a fast line-scanning Zeiss LSM7 system (Oberkochen, Germany), equipped with a 63× oil immersion objective.

Reconstituted Human Blood

For reconstitution of human blood, platelets were isolated, and platelet count was adjusted to 250×10^6/mL with HEPES buffer pH 7.45. Washed erythrocytes were obtained by centrifugation of citrate anticoagulated blood at 250*g* for 15 minutes. For the removal of remaining plasma and platelets, erythrocytes were washed 2× with HEPES buffer pH 7.45.[25] Reconstituted

blood (washed platelets [250×10^6/mL], washed erythrocytes [Hct=40%], 100 µg/mL human fibrinogen, 7.5 µg/mL AF546-conjugated human fibrinogen, 2 mmol/L $CaCl_2$, and 2 mmol/L $MgCl_2$) was incubated with vehicle or indicated peptide (50 nmol/L) in the presence of 7 µmol/L sitagliptin for 10 minutes. Glass coverslips were coated with 2 µL microspots of collagen type I (100 µg/mL) plus VWF (von Willebrand factor; 50 µg/mL). Whole blood thrombus formation was performed as described above.

Light Transmission Aggregometry

Washed platelets (25×10^7/mL, 2 mmol/L $CaCl_2$) or PRP were preincubated with sitagliptin (7 µmol/L) in combination with GLP-1(7-36) (50 nmol/L) or vehicle for 10 minutes at room temperature. Platelet aggregation was induced by a submaximal concentration of collagen type I and measured under constant stirring at 37°C using a lumi-aggregometer (Chronolog Corporation; Havertown, PA).

Flow Cytometry

Human washed platelets (50×10^6/mL) and citrate anticoagulated whole blood were treated with vehicle, 50 nmol/L GLP-1(7-36) or 50 nmol/L GLP-1(9-36) in the presence of 7 µmol/L sitagliptin for 10 minutes. Subsequently, platelet activation was induced by 1 µg/mL CRP-XL. Activated platelets were labeled with FITC-labeled PAC-1(1:20 [washed platelets], 1:25 [whole blood]; to analyze integrin activation), PE-labeled P-selectin (1:40 [washed platelets], 1:25 [whole blood]) and AF-647 labeled TLT-1 (TREM-like transcript 1; 1:25 [washed platelets], 1:25 [whole blood]). Integrin $\alpha_{IIb}\beta_3$ activation, P-selectin, and TLT-1 expression were assessed using flow cytometry (BD Canto II). Data were analyzed using FlowJo software 10.6.1.

Statistics and Heat Mapping

After testing for normality and equal variance, data were analyzed as appropriate, using a one-sample *t* test with a Bonferroni correction for multiple comparisons, a Wilcoxon matched-pairs signed-rank test (nonparametric) with a Bonferroni correction for multiple comparisons, a one-way ANOVA with Dunnett multiple comparisons test or a two-way ANOVA with a Sidak multiple comparisons test. GraphPad Prism 6 Software was used throughout. $P<0.05$ were considered to be statistically significant.

Mean values of whole blood thrombus formation assays were averaged per parameter and per surface and scaled over a range from 0 to 10 per parameter. Univariate scaled heatmaps were generated using the program R.[17,26] Where indicated, data were transformed into subtraction heatmaps to compare to control conditions. Filtering of the subtraction arrays was according to conventional large effect sizes (Cohen's d ≥0.8).[27]

RESULTS

Platelets Harbor High DPP-8/9 and Minimal DPP-4 Activity Compared to Plasma

We first determined DPP-4-like enzyme activity in platelet lysates and in releasates of activated platelets using the DPP-IV-Glo protease assay. This assay quantifies DPP-4-like N-terminal X-Pro dipeptide cleavage at a defined pH, which can be caused by DPP-4 or by the related DPP-4 activity and/or structure homolog (DASH) proteases, DPP-8 and DPP-9. As assessed in lysates as well as platelet releasates, platelet stimulation with thrombin, ionomycin or CRP-XL could not trigger an increase in DPP-4-like activity (Figure 1A and 1B). To identify the active DPP enzyme, enzymatic measurements were repeated in the presence of sitagliptin (to block DPP-4) or 1G244 inhibitor (to block DPP-8/DPP-9) at concentrations optimized for enzyme selectivity (Figure IA and IB in the online-only Data Supplement). This analysis revealed that the DPP activity in lysed platelets was because of DPP-8/9 and not to DPP-4 (Figure 1A). In platelet releasates, DPP-4-like activity was because of DPP-4, with only minor levels of DPP-8/9 activity measurable (Figure 1B). Absolute quantification demonstrated in washed platelets a DPP-8/9 activity of 82±6 mU/g protein, whereas the DPP-4 activity was limited to 3±1 mU/g (Figure 1C). In contrast, platelet-free plasma revealed a high enzyme activity of DPP-4 (113±16 mU/g) but no enzymatically active DPP-8/9.

Together, these results indicate that the DPP-4-like activity in platelets derives from DPP-8/9 and not DPP-4, whereas platelet-free plasma displays high DPP-4 but no DPP-8/9 activity.

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

To examine the effect of DPP-4 on platelet responses, we studied 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 assessed by multicolor microscopy (Figure 2A). Based on the microscopy images, a subtraction heatmap consisting of scaled values of 8 parameters of thrombus formation was generated and filtered for large effect sizes (Cohen's d ≥0.8). This revealed 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 2B, Table III in the online-only Data Supplement). Along the same line, treating wild-type mice for 5 days with the DPP-4 inhibitors linagliptin and sitagliptin, markedly 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 2C, Table III in the online-only Data Supplement).

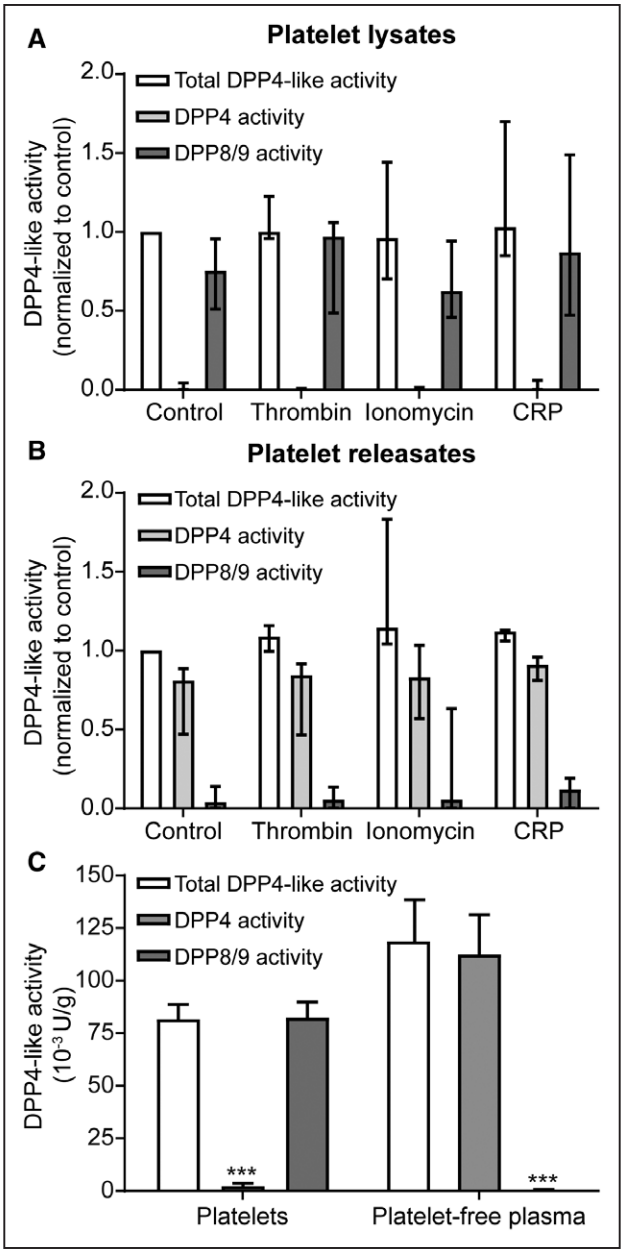

Figure 1. High DPP (dipeptidyl peptidase)-8/9 activity in platelets and high DPP-4 activity in plasma.
A and **B**, Washed human platelets (1×10^7/mL) were stimulated with thrombin (1 U/mL), ionomycin (10 µmol/L), or CRP-XL (collagen-related peptide; 5 µg/mL) at 37°C for 45 minutes, as indicated. As a negative control, platelets were treated with prostacyclin (0.1 µg/mL). DPP-4-like activity, specific DPP-4 activity (in the presence of DPP-8/9 inhibitor 1G244, 7 µmol/L), and DPP-8/9 activity (in presence of DPP-4 inhibitor sitagliptin, 7 µmol/L). Activity measurement in (**A**) platelet lysates and (**B**) platelet releasates using the DPP-IV-Glo protease assay. DPP activity was expressed relative to total DPP-4-like activity in prostacyclin-treated platelets (ctrl), and displayed as medians with interquartile range (IQR); n=6–8. Wilcoxon matched-pairs signed-rank test (stimulated vs ctrl), with Bonferroni correction for multiple comparisons. **C**, DPP-4-like activity and specific DPP-8/9 and DPP-4 activities in lysates from resting platelets and in platelet-free plasmas, quantified as described[21]; n=5–6. Data are displayed as means ± SD; two-way ANOVA with Sidak multiple comparisons test, ***P<0.001.

Platelet counts were not affected by DPP-4 deficiency or inhibition and neither were white nor red blood cell counts (Table VI in the online-only Data Supplement). Treating *Dpp4*$^{-/-}$ mice with linagliptin or sitagliptin could not further reduce responses of platelets under flow (Figure 2C, Table III in the online-only Data Supplement), indicating that the inhibitory effects of linagliptin and sitagliptin on the thrombotic process are mediated by interference with DPP-4.

As previous research suggested that GLP-1R activation reduces platelet activation at least partially by raising cAMP levels,[13] we also assessed thrombus formation in the presence of the stable prostacyclin analog iloprost, which elevates platelet cAMP levels via Gs stimulation.[28] Compared to DPP-4 deficiency, iloprost pretreatment led to a more severe reduction in essentially all parameters of thrombus formation. Upon additional DPP-4 deficiency, we observed a minor further decrease in platelet adhesion and integrin activation on collagen I (Figure IIB through IID and Tables III and IV in the online-only Data Supplement). DPP-4 deficiency did not alter thrombus formation on a less activating collagen III surface in either the absence or presence of iloprost (Figure IIA through IIC and Table IV in the online-only Data Supplement). Together, these results indicate that genetic absence, as well as inhibition of DPP-4, leads to a reduction in collagen I-dependent thrombus formation under flow, although to a lesser degree than caused by cAMP elevation with iloprost.

With the GLP-1R agonist exendin-4 previously shown to reduce platelet aggregation,[13,14] we next investigated whether GLP-R1 signaling may explain the observed effects on platelet responses in DPP-4 knockout mice. Analysis of *Glp1r* expression in highly pure, isolated platelets by droplet digital polymerase chain reaction analysis revealed low but detectable levels of *Glp1r* mRNA transcripts in mouse platelets (Figure IIIA through IIIC in the online-only Data Supplement). Thus, we assessed whether GLP-1R blocking with exendin-9 could increase platelet responsiveness in *Dpp4*$^{-/-}$ mice. In flow assays over collagen I, blood from *Dpp4*$^{-/-}$ mice showed comparable platelet deposition (37.67±8.28 [*Dpp4*$^{-/-}$] versus 43.03±15.02 [*Dpp4*$^{-/-}$ + exendin-9], P=0.29, n=10–13) and multilayer thrombus formation (13.67±6.27 [*Dpp4*$^{-/-}$] versus 16.86±11.16 [*Dpp4*$^{-/-}$ + exendin-9], P=0.39, n=10–13; mean±SD) in the absence versus presence of exendin-9. Also, adding exendin-9 to *Dpp4*$^{-/-}$ blood did not alter parameters of thrombus morphology (3.79±0.68 [*Dpp4*$^{-/-}$] versus 4.21±0.83 [*Dpp4*$^{-/-}$ + exendin-9], P=0.19, n=11–13), contraction (1.86±0.78 [*Dpp4*$^{-/-}$] versus 2.21±0.77 [*Dpp4*$^{-/-}$ + exendin-9], P=0.20, n=12–13) and multilayer scores (1.89±0.61 [*Dpp4*$^{-/-}$] versus 2.17±0.85 [*Dpp4*$^{-/-}$ + exendin-9], P=0.35, n=11–13). Furthermore, differently than observed for *Dpp4*$^{-/-}$ mice, GLP-1R deficiency did not alter platelet

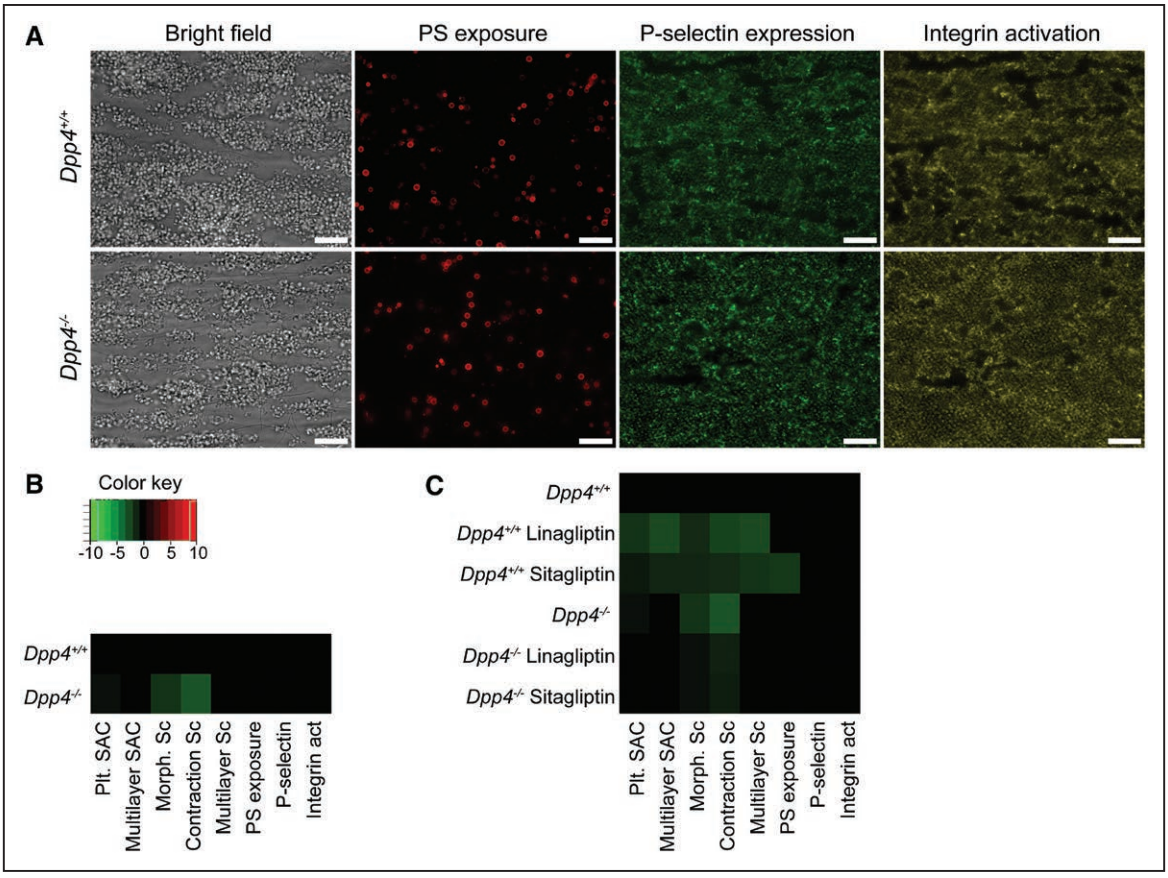

Figure 2. Murine DPP-4 (dipeptidyl peptidase 4) deficiency impairs thrombus formation under flow.
Blood from *Dpp4*$^{-/-}$ or wild-type control mice was perfused over collagen I microspots at 1000/s. Where indicated, mice were fed for 5 d a normal laboratory diet supplemented with linagliptin or sitagliptin. Thrombi formed were poststained with fluorescein isothiocyanate (FITC)-labeled anti-P-selectin mAb (secretion), PE-labeled 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.

adhesion, aggregation, activation, or thrombus structure parameters on collagen I and only induced an increase in phosphatidylserine exposure (Figure IVA through IVC and Tables III and IV in the online-only Data Supplement). On a collagen III surface, on which we had not observed effects of DPP-4 deficiency (Figure II and Table IV in the online-only Data Supplement), GLP-1R deficiency increased parameters of thrombus morphology, contraction, and multilayer score (Figure IVA through IVC and Table IV in the online-only Data Supplement). Whereas the addition of GLP-1(7-36) did not affect thrombus formation in wild-type mice under the investigated conditions, it still could annul the effects of GLP-1R deficiency on collagen III induced thrombus formation (Figure IVA through IVC and Table IV in the online-only Data Supplement), indicating GLP-1 to reduce platelet responses independently of GLP-1R.

In summary, these data indicate that deficiency or blocking DPP-4 suppresses thrombus formation under flow in mouse blood without a main relevance of GLP-1R on platelets.

Native GLP-1(7-36) but Not DPP-4-Cleaved GLP-1(9-36) Reduces Platelet Aggregation in Human Whole Blood Under Flow

Next, we tested the effects of a short-term treatment of human blood samples with the DPP-4 inhibitor sitagliptin. Sitagliptin did not affect collagen I-induced thrombus formation under flow (Figure V in the online-only Data Supplement), while it efficiently inhibited DPP-4 (data not shown). These results hence raised the hypothesis that the reduced thrombus formation, observed by in vivo DPP-4 inhibition in mice, is due to an accumulation of noncleaved over cleaved DPP-4 substrates, rather than by a direct DPP-4-mediated enzymatic effect on the thrombus formation parameters.

Given that the clinically applied DPP-4 inhibitors increase the plasma ratio of native GLP-1(7-36) over DPP-4-cleaved GLP-1(9-36), we directly compared the effects of GLP-1(7-36) versus GLP-1(9-36) peptides on human thrombus formation under a variety of flow conditions, that is, low (venous), intermediate (large arterial),

or high (small arterial) wall-shear rates. The heatmap of GLP-1(7-36) and GLP-1(9-36)-mediated effects on parameters of thrombus formation, obtained from series of microscopy images, indicated a substantial overall reduction of thrombus formation by GLP-1(7-36) under flow. Notably, this reduction was seen at all shear-flow conditions, from low (150/s) and intermediate (1000/s) to high (1700/s; Figure 3A, Figure VI and Table V in the online-only Data Supplement). For quantification, subtraction heatmaps were generated in which the changes were filtered for large effect sizes (Cohen's d ≥0.8). This analysis revealed reductions especially in aggregation-related parameters (multilayer thrombus SAC, multilayer, morphological, and contraction scores), whereas the typical platelet activation markers P-selectin and activated integrin (Figure 3B, Table V in the online-only Data Supplement), as well as TLT-1[29] (data not shown), were less affected.

In addition, we investigated the effects of GLP-1(7-36) on thrombus formation on co-infusion with stable ADP. This resulted in a clear reduction in platelet deposition (30.17±8.69% [control] versus 25.25±14.38% GLP-1(7-36)) as well as platelet aggregation (21.35±4.06% [control] versus 8.76±3.54% [GLP-1(7-36)]; Figure 3C and 3D, Figure VIC and Table V in the online-only Data Supplement). In sharp contrast, equal concentrations of the DPP-4-cleaved GLP-1(9-36) were unable to reduce thrombus formation under flow but instead triggered minor increases in adhesion-related parameters (multilayer SAC at 150/s; platelet SAC at 1000/s with ADP co-infusion; Figure 3B and 3D, Table V in the online-only Data Supplement).

In summary, native GLP-1(7-36), but not DPP-4-cleaved GLP-1(9-36), reduces platelet aggregation in human whole blood under flow.

Platelet-Inhibitory Effect of Native GLP-1(7-36) Requires Flow but Not Plasma Factors or Leukocytes

To assess the effects of GLP-1 in further detail, we examined aggregation responses of slowly stirred suspensions of human platelets using light transmission aggregometry. Strikingly, and in contrast to the clear GLP-1-mediated effects on whole blood thrombus formation, neither GLP-1(7-36) nor GLP-1(9-36) did affect collagen-induced platelet aggregation responses in the stirred suspensions (fold change for GLP-1(7-36): 1.13±0.13 and 0.94±0.04 in washed platelets and PRP, respectively, versus 0.74±0.03 in whole blood; Figure 4). Furthermore, neither GLP-1(7-36) nor GLP-1(9-36) affected CRP-XL-induced platelet activation markers, as quantified by flow cytometric analysis of integrin $\alpha_{IIb}\beta_3$ activation, when assessed in washed platelets or in whole blood (Figure 4, Figure VIIA and VIIB in the online-only Data Supplement). Similarly, the CRP-XL-induced expression of P-selectin and TLT-1 on platelets was unaltered in the presence of GLP-1(7-36) or GLP-1(9-36) in either washed platelets or whole blood (Figure VIIA and VIIB in the online-only Data Supplement). Along the same line, neither GLP-1(7-36) nor GLP-1(9-36) altered the phosphorylation state of AKT (AKT serine/threonine kinase), ERK (extracellular signal-regulated kinase) 1/2, or SYK (spleen-associated tyrosine kinase) in human platelets on thrombin, ADP, or CRP-XL stimulation (Figure VIII in the online-only Data Supplement). However, GLP-1(7-36) still reduced thrombus size and contraction under flow even in the absence of plasma factors, as analyzed using reconstituted blood samples containing only washed platelets, erythrocytes, and fibrinogen (Figure 5). Combined, these results underline that GLP-1(7-36) depends on flow conditions but does not require plasma factors for its inhibitory effect on platelets.

Triggered by these GLP-mediated effects on human platelet responsiveness, we analyzed *GLP1R* expression in preparations of highly pure, human platelets. No *GLP1R* transcripts could be detected by droplet digital polymerase chain reaction in human platelets, despite *GLP1R* expression in human pancreas as positive control (Figure IIID through IIIF in the online-only Data Supplement). Thus, these data indicate that platelet GLP-1R does not play a main role in the GLP-mediated effects observed under flow.

Recorded microscopic images from collagen I surfaces suggested that GLP-1(7-36) suppressed the 3-dimensional buildup of thrombi under flow conditions. To confirm this, we quantified thrombus volumes by recording confocal z-stacks of thrombi consisting of $DiOC_6$-labeled platelets. Markedly, the addition of GLP-1(7-36) reduced thrombus volume and height by 32±8% and 23±9%, respectively (mean±SD; Figure 6A and 6B), with an overall smaller thrombus area at each confocal plane (Figure 6C and 6D). In line with previous observations, the DPP-4-cleaved GLP-1(9-36) did not affect thrombus volume or height (Figure 6).

Altogether, these results reveal a mechanism in which, in vivo, plasma-derived DPP-4 activity promotes thrombus formation by cleaving native GLP-1(7-36) to GLP-1(9-36). The cleaved GLP-1(9-36) can no longer suppress thrombus formation in whole blood. In the absence of DPP-4 activity—either by genetic deficiency or by long-term inhibitory treatment—the native, uncleaved GLP-1 accumulates in the blood and continues to suppress the flow-dependent formation of thrombi, independent of plasma factors and without a main relevance of GLP-1R on platelets.

DISCUSSION

In this article, we provide novel clarification for the antithrombotic effect of DPP-4 inhibition, acting through suppressed cleavage of GLP-1(7-36) to GLP-1(9-36). Our findings demonstrate that inhibition or genetic deficiency of DPP-4 in mice results in reduced thrombus formation

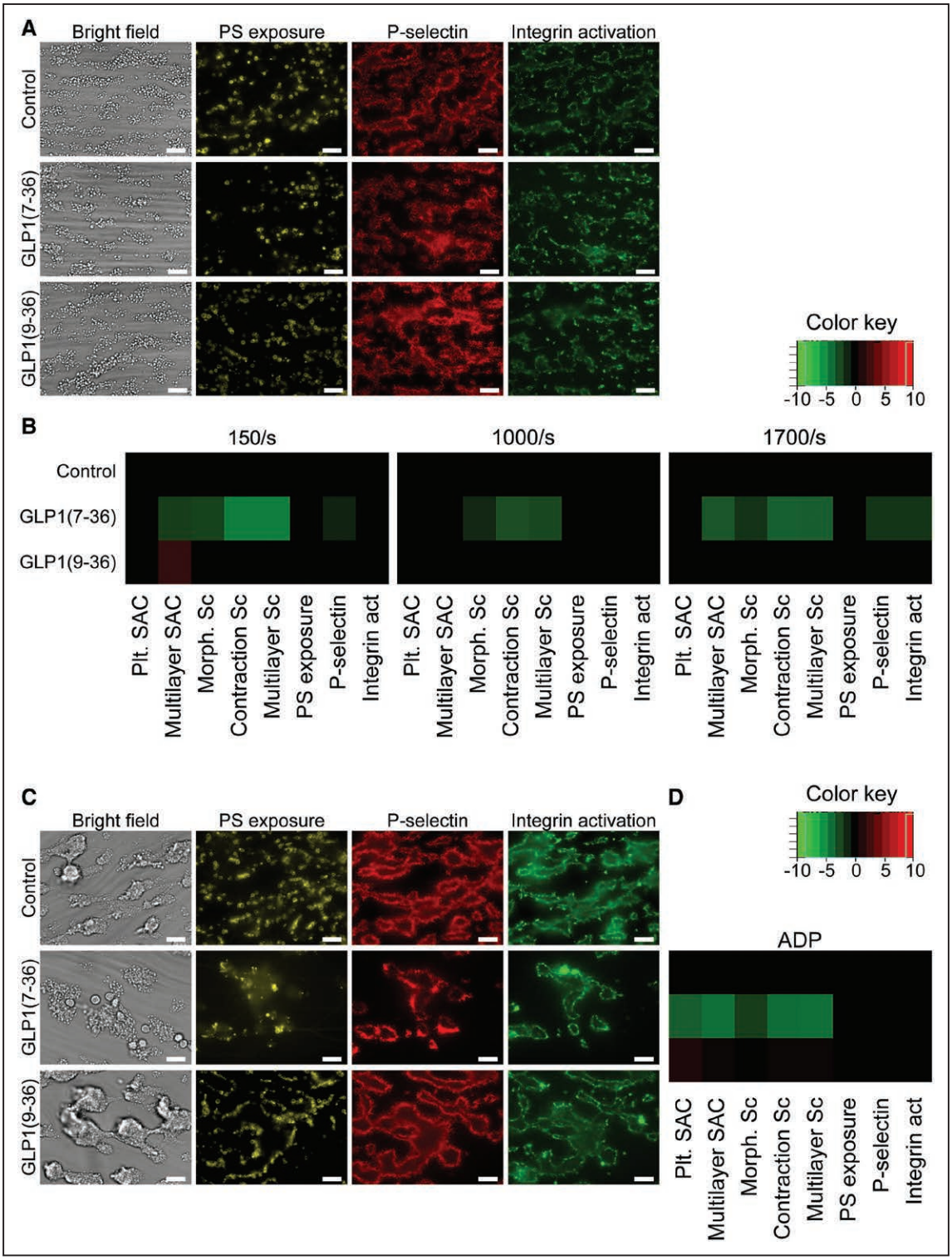

Figure 3. GLP-1 (glucagon-like peptide 1 [7-36]) suppresses human whole blood thrombus formation regardless of wall-shear rate. Human blood was preincubated with GLP-1(7-36) (50 nmol/L), GLP-1(9-36) (50 nmol/L) or vehicle control for 10 min. Sitagliptin (7 μmol/L) was present in all conditions. **A** and **B**, After recalcification, samples were perfused over collagen I microspots at indicated wall-shear rates. Thrombi formed were poststained for phosphatidylserine (PS) exposure, P-selectin expression, and integrin activation. **A**, Representative brightfield and fluorescence images at wall-shear rate of 1000/s. **B**, Subtraction heatmap of scaled parameter values, compared to means of controls; filtered for differences with large effect size (Cohen's ≥0.8); n=12 for 1000/s; n=4 for 150/s and 1700/s. **C** and **D**, Blood samples were coperfused with 2-methylthioadenosine diphosphate (2MeADP; 1 μmol/L) over collagen 1000/s. **C**, Representative brightfield and fluorescence images (n=4). **D**, Subtraction heatmap of scaled values, compared to means of controls; filtered for large effect size as above. Scale bars =20 μm. Figure VI in the online-only Data Supplement shows representative images for thrombus formation at shear rates 150/s and 1700/s. SAC indicates surface area coverage.

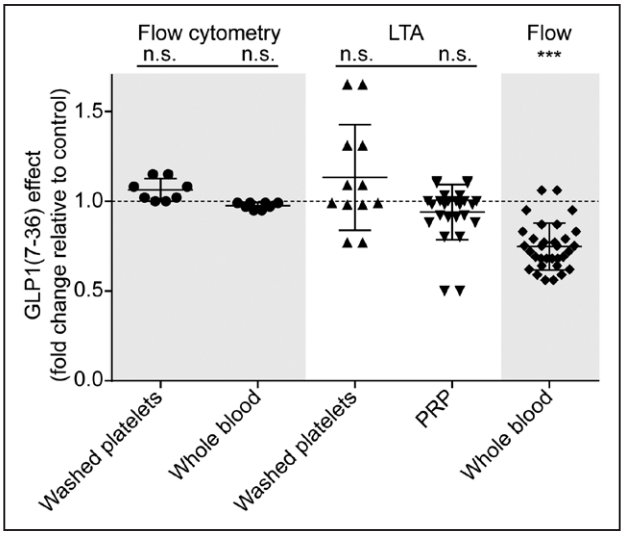

Figure 4. GLP-1 (glucagon-like peptide 1 [7-36]) impairs collagen-induced platelet aggregation only in flowed whole blood.

Effect of GLP-1(7-36) (50 nmol/L) on (1) CRP-XL (collagen-related peptide)-induced platelet activation in washed platelets and whole blood as assessed by flow cytometry, analyzing integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression (n=4) and on (2) collagen-induced aggregate formation of washed platelets, platelet-rich plasma (PRP), and thrombus formation under flow, as indicated. Collagen-induced maximal aggregation of slowly stirred washed platelets (n=6) and PRP (n=14) was measured using a lumi-aggregometer (light transmission aggregometry; LTA). Collagen-dependent aggregate formation under flow at 1000/s was measured from overall aggregation parameters (multilayers, morphological, contraction, and multilayer scores; n=16). Present was 7 µmol/L sitagliptin in all conditions. Effects of GLP-1(7-36) are expressed as fold changes relative to vehicle control and are displayed as dot plots with indication of means ± SD. One-sample *t* test with Bonferroni correction for multiple comparisons. ***$P<0.001$; ns =not significant.

under flow. This regulation could be confirmed in human whole blood samples by the native peptide GLP-1(7-36), that is, a main substrate of DPP-4. In this case, the elevation of GLP-1(7-36) but not of the DPP-4-cleaved GLP-1(9-36) resulted in the formation of smaller and less contracted thrombi formed on collagen, at both venous and arterial flow conditions. The inhibitory effect of GLP-1(7-36) on platelet responsiveness required flow conditions, but not plasma factors, leukocytes or GLP-1R on platelets. In contrast to native GLP-1(7-36), the DPP-4-cleaved form GLP-1(9-36) triggered minor increases in platelet responsiveness in flowed whole blood.

Several earlier studies have shown that DPP-4 is expressed by different cell types within the blood and vasculature, including T lymphocytes and endothelial cells,[3,30] but platelet DPP-4-like activity had not been studied in detail before. Here, we indicate that the DPP-4-like activity, measurable in platelets, is primarily due to the DASH proteases DPP-8/9 and not to DPP-4. These results are in line with recent data reporting that megakaryocytes and platelets do not harbor detectable DPP-4 enzyme activity[14] and express the DPP-4-family members DPP-2, DPP-3 and DPP-9 but not DPP-4 according to proteomics analyses.[31] Accordingly, residual DPP-4 activity detected in platelet releasate may have been derived from plasma remnants, for example, present in the open canicular system of platelets. Furthermore, we could confirm that plasma reveals high DPP-4 activity.

When DPP-4 activity was deficient in mice, either by long-term in vivo administration of linagliptin or sitagliptin or by genetic deficiency, we observed marked reductions in collagen I-dependent platelet responses under flow. In contrast, short-time DPP-4 inhibition did not influence whole blood thrombus formation, indicating that the platelet responds to the accumulation of uncleaved over cleaved DPP-4 substrates, rather than to the DPP-4 enzymatic activity per se. The observation that in vivo treatments with linagliptin or sitagliptin suppressed platelet responses in wild type but not in *Dpp4*$^{-/-}$ mice indicates that the inhibitory effects of these drugs are mediated through DPP-4 itself and not through off-target (non–DPP-4 related) effects. In line with this conclusion, a previous study revealed reduced endotoxemia-induced thrombosis in mice with a *Dpp4* deficiency or treated with the DPP-4 inhibitor linagliptin.[13]

One of the best described DPP-4 substrates is GLP-1(7-36), which is cleaved by DPP-4 to GLP-1(9-36).[3] Physiologically, the half-life of GLP-1(7-36) is short (1.5–5 minutes).[32] Accordingly, the stable, synthetic GLP-1(7-36)-analog liraglutide (97% amino acid sequence identity to GLP-1[7-36]) and the peptide exenatide (a synthetic form of the GLP-1R agonist exendin-4, with 50% sequence identity to GLP-1(7-36)[15]) are of improved efficacy in the clinical therapy of hyperglycemia. As an alternative, DPP-4 inhibitors are clinically used to rise the endogenous levels of native, uncleaved GLP-1.[33] In this article, we specifically focused on the platelet effects of the physiological, uncleaved versus DPP-4-cleaved GLP-1 peptides. In accordance with the phenotypes of DPP-4 deficiency and long-term in vivo DPP-4 inhibition, we could establish that increased levels of native GLP-1(7-36), but not of cleaved GLP-1(9-36), reduced whole blood thrombus formation under flow.

Our findings indicate that the largest effects of GLP-1(7-36) supplementation are observed in the presence of shear flow. In flowed human blood, GLP-1(7-36) addition caused a markedly decreased thrombus volume and height. In contrast, the peptide was unable to reduce collagen-induced platelet aggregation in slowly stirred suspensions of washed platelets or PRP, and it did not affect platelet activation markers in flow cytometry analyses of washed platelets or whole blood. In line with a report of Barale et al[34] in which GLP-1(7-36) alone was unable to reduce collagen- or arachidonic acid-induced platelet aggregation, our data suggest that shear stress is needed to achieve the GLP-1(7-36)-mediated platelet inhibition. As VWF-GPIb interactions

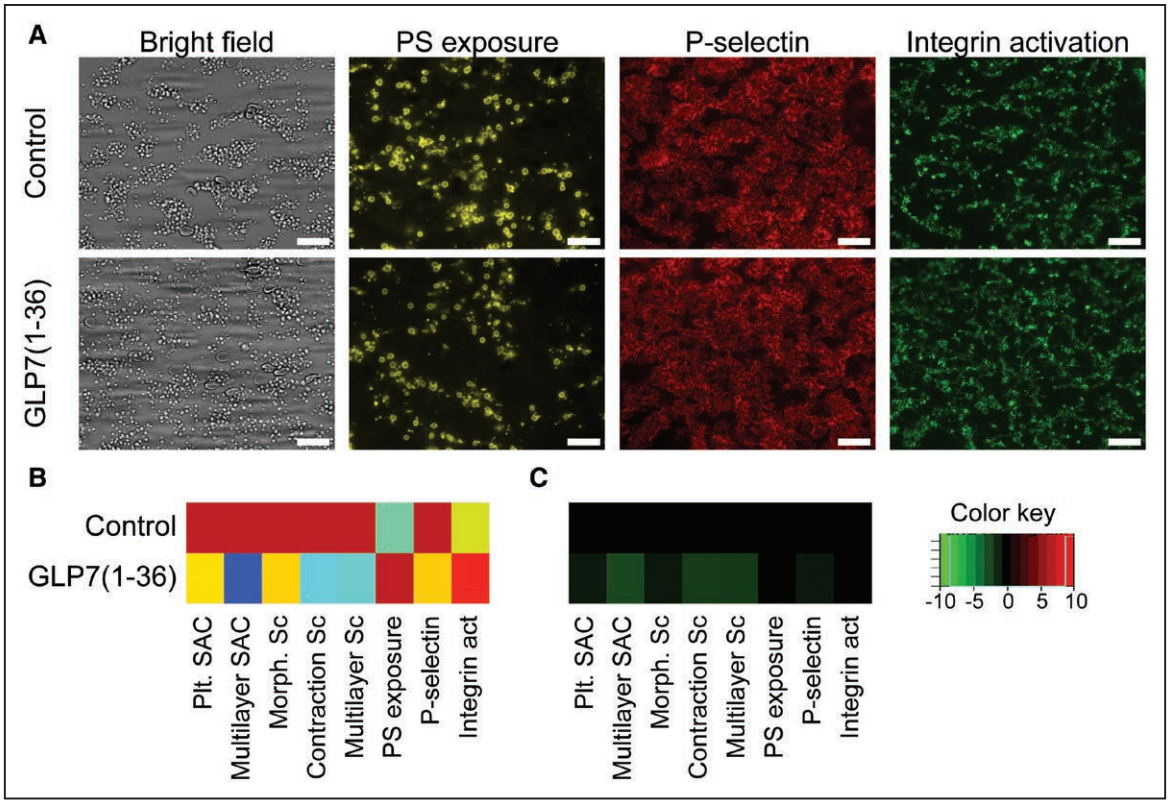

Figure 5. Effects of GLP-1 (glucagon-like peptide 1 [7-36]) on thrombus formation under flow persist in absence of plasma factors and leukocytes.

Reconstituted human blood (containing washed platelets, erythrocytes, and fibrinogen) was incubated for 10 min with vehicle or 50 nmol/L GLP-1(7-36) in the presence of 7 µmol/L sitagliptin and perfused over a VWF (von Willebrand factor)-collagen type I surface at 1000/s. Thrombi were poststained for P-selectin expression and phosphatidylserine (PS) exposure. **A**, Representative brightfield and fluorescence images. **B**, Heatmap of univariate scaled (0–10) values for control or GLP-1(7-36) treatment. **C**, Subtraction heatmap of scaled parameter values, compared to means of control, filtered for differences with large effect size (Cohen's d ≥0.8). Scale bar =20 µm. n=4. SAC indicates surface area coverage.

are essential for high shear mediated platelet adhesion, we also examined a role of GLP-1(7-36) under conditions of low/venous shear rate (150/s), where platelet adhesion and activation occurs independently of the VWF-GPIb axis.[17] However, at all flow shear rates examined (150, 1000, and 1700/s), we observed a similar reduction of platelet aggregation parameters, strongly indicating that the effect of GLP-1(7-36) is independent of VWF. Literature further indicates that the GLP-1(7-36) analog liraglutide also could not reduce collagen- or arachidonic acid-induced platelet aggregation in stirred PRP,[34] whereas much higher levels (10–100 µmol/L) of the GLP-1(7-36) analog liraglutide were required to reduce ADP-induced platelet aggregation.[13]

Furthermore, our study revealed that the inhibitory effect of GLP-1(7-36) does not require plasma factors or leukocytes, as revealed by flow assays with reconstituted blood containing only washed platelets, washed erythrocytes and fibrinogen, and occurs without a main role for GLP-1R on platelets. In this context, expression of GLP-1R on platelets has been an intense matter of debate, in particularly also related to the lack of antibodies with high sensitivity and verified specificity for human as well as mouse GLP-1R.[18,35] On the one hand, Western blot and flow cytometer experiments have demonstrated expression of GLP-1R on human platelets as well as on mouse platelets.[13,14,34] On the other hand, proteomic analyses could not detect GLP-1R in human platelets[31] or with only 2% sequence coverage,[36] and also only low *GLP1R* mRNA levels were detected in the megakaryocyte platelet lineage.[37] Using highly sensitive droplet digital polymerase chain reaction, we could not detect *GLP1R* expression in human platelets, in line with transcriptomics data from the Blueprint Consortium (http://dcc.blueprint-epigenome.eu/#/home) revealing normalized expression values for *GLP1R* to be negligible in human platelets (log2(fpkm)=0.0005) compared to typical platelet receptors (eg, GPIbα: log2(fpkm)=9.26). Alongside, we could also detect only very low levels of *GLP1R* in human PBMCs, in line with transcriptomics data within the Human Protein Atlas database as well as provided by Schmiedel et al,[38] revealing low *GLP1R* expression only in natural killer cells and specific T-cell subsets (≈0.2–5.2 transcripts per million). Our transcript analysis of mouse platelets did detect *Glp1r* mRNA. However, *Dpp4*$^{-/-}$ mice did not show significant differences

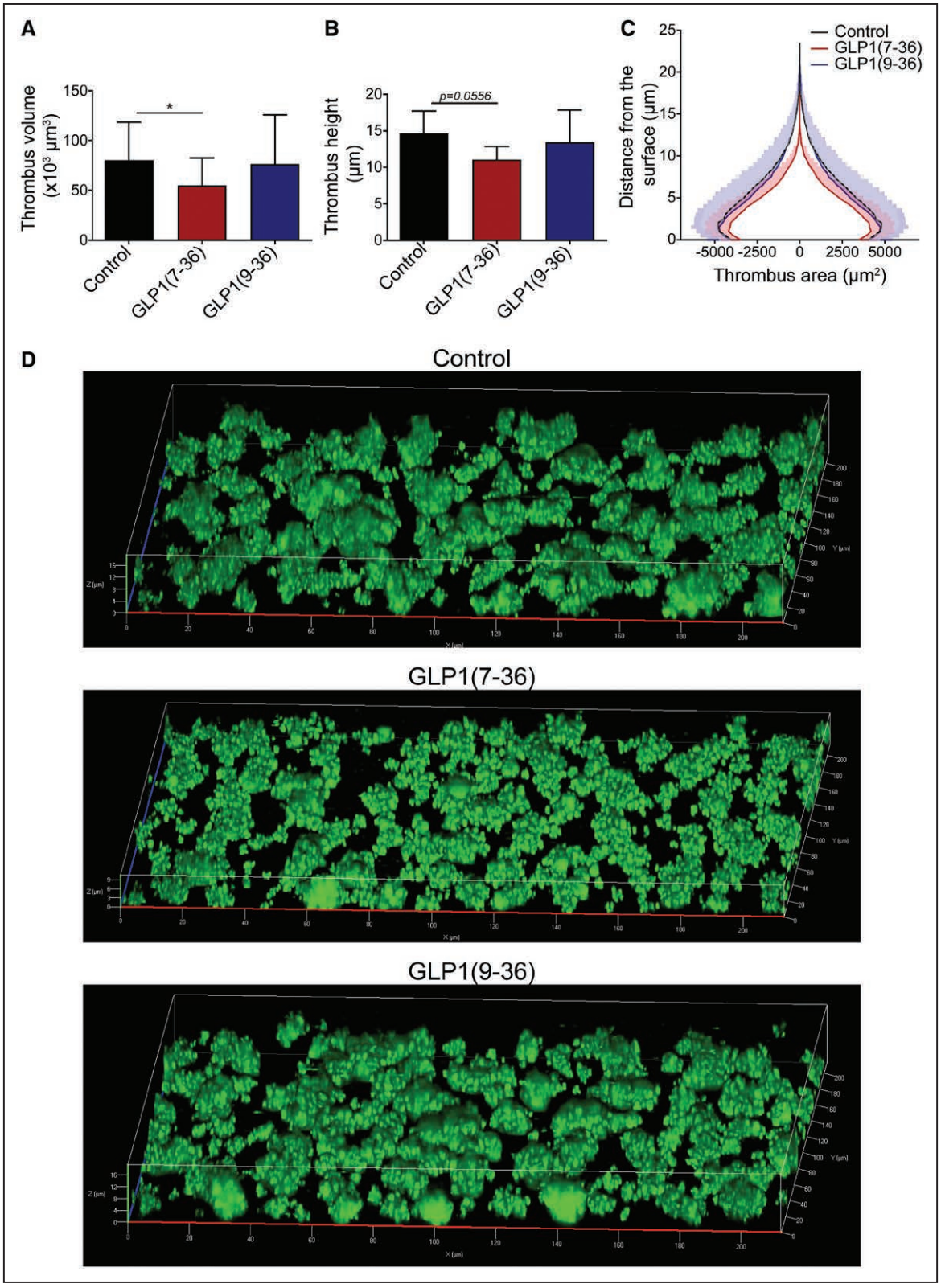

Figure 6. GLP-1 (glucagon-like peptide 1 [7-36]) reduces thrombus size under flow.
Human blood was incubated for 10 min with vehicle, GLP-1(7-36) (50 nmol/L) or GLP-1(9-36) (50 nmol/L) in the presence of 7 µmol/L sitagliptin. Platelets in the blood were prelabeled with $DiOC_6$. After flow perfusion at 1000/s, z-stacks were recorded of thrombi formed on collagen I microspots. **A** and **B**, Microscopy images analyzed for overall thrombus volume (**A**) and thrombus height (**B**). Data are displayed as means ± SD, n=4. One-way ANOVA with Dunnett multiple comparisons test vs vehicle; *P<0.05. **C**, Graphical representation of mean thrombus area (µm²) with SD (shaded area) for indicated distances from collagen surface. **D**, Representative z-stacks.

in platelet deposition and aggregation in whole blood flow assays upon treatment with the GLP-1R antagonist exendin-9. Also, collagen I-induced thrombus formation under flow was unaltered in *Glp1r*$^{-/-}$ mice. Combined, these findings argue against a role of platelet GLP-1R in GLP-1-mediated modulation of both human and mouse platelet responses and support the involvement of another receptor or receptor-independent mechanism instead. This is in line with recent findings of Barale et al,[34] who showed that GLP-1(7-36), as well as its synthetic homolog liraglutide, increased the inhibitory effects of the NO donor sodium nitroprusside on human platelet aggregation independently of GLP-1R. Also, renoprotective and immune-regulatory capacities were recently identified for native, intact GLP-1, which could not be fully recapitulated by GLP-1R agonists.[39] Combined, these studies, as well as our own article, reveal the increasingly appreciated importance of GLP-1R-independent actions of native, intact GLP-1. In relation to the antithrombotic effects previously observed for the GLP-1R agonist exenatide upon laser injury of the cremaster arterioles in mice, a role for non-bone marrow-derived cells was identified, with a potential involvement of the endothelium suggested.[14]

A recent international consensus article compared clinical trials on effects of DPP-4 inhibitors and GLP-1R agonists on cardiovascular outcomes in patients with diabetes mellitus. A potential of cardiovascular protection was revealed for true homologs of native GLP-1 (liraglutide and semaglutide, showing 97% and 94% sequence identity to GLP-1, respectively) and this independent of glucose control. In contrast, the GLP-1R agonists lixasenatide and exenatide, both sharing sequence similarity to exendin-4 (which is only ≈50% identical to GLP-1[15]), were not, or only to a minor extent, associated with cardioprotection.[40] In this context, our data contribute a mechanistic explanation for such protection, in that native, intact GLP-1 exerts platelet-inhibitory effects without a main relevance of GLP-1R on platelets. Especially in conditions of enhanced thrombus formation, such as in our study with ADP co-infusion, effects of increased GLP-1(7-36) over GLP-1(9-36) ratios were strikingly beneficial. In this context, native GLP-1(7-36) could be regarded as natural suppressor of platelet aggregation and thrombus growth under physiological flow conditions. Since overall, DPP-4 inhibitors could not provide cardioprotection in clinical trials,[40] it can currently not be excluded that simultaneous stabilization of other DPP-4 substrates (eg, substance P or fibrin[41,42]) may interfere with GLP-1(7-36) mediated antiplatelet effects of DPP-4 inhibitors in patients, thus requiring further investigation.

In conclusion, our data provide novel evidence that DPP-4 inhibition and native GLP-1(7-36) can reduce whole blood thrombus formation at both venous and arterial flow shear rates, resulting in the formation of smaller and less contracted thrombi and this without a main relevance of GLP-1R on platelets. Furthermore, the crucial role of flow in GLP-1(7-36)–induced platelet effects observed in this study underlines the need to investigate thrombus formation in patients with diabetes mellitus treated with DPP-4 inhibitors and GLP-1 mimetics specifically under physiological flow conditions.

ARTICLE INFORMATION

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Disclosures

J.W.M. Heemskerk is a co-founder and shareholder of FlowChamber. The other authors report no conflicts.

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