VOL. 7, NO. 7, 2022
ISSN 2452-302X

Letter

RESEARCH LETTER

Glucagon-Like Peptide-1 Receptor Regulates Thromboxane-Induced Human Platelet Activation

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are approved to reduce cardiovascular disease in those with type 2 diabetes mellitus (T2DM). Longer acting GLP-1RAs, liraglutide and semaglutide, demonstrate a significant reduction in major cardiovascular events not observed in short-acting GLP-1RAs or dipeptidyl peptidase-4 inhibitors.[1] The mechanism by which GLP-1RAs improve cardiovascular risk in T2DM is likely multifactorial with indirect effects on platelet aggregation postulated.[1]

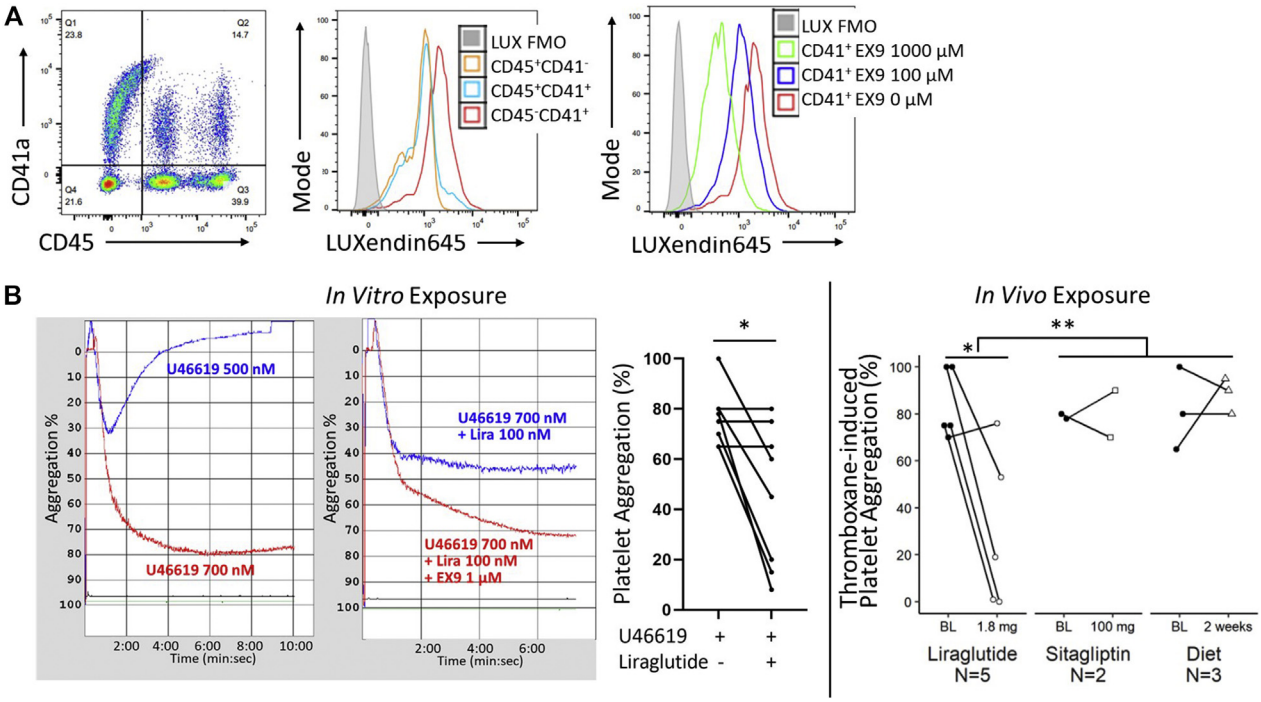
Platelet activation, independent from hemostasis, contributes to inflammation in the cardiovascular system. Prior in vitro studies suggest that GLP-1RAs reduce thrombin-induced platelet aggregation via GLP-1R-independent mechanisms and enhance nitric oxide inhibition of adenosine-, collagen-, and arachidonic acid-induced platelet aggregation in normocholesterolemic states, but no evidence shows a direct GLP-1R effect on platelets.[2] Thromboxane A_2 (TX) is a proinflammatory mediator in cardiovascular disease, particularly in T2DM.[3] Patients with T2DM demonstrate elevated platelet TX production and increased urinary TX metabolites. TX is generated by endothelial cells, activated platelets, and macrophages. It binds the TX receptor on platelets to enhance activation and aggregation. We hypothesized that the GLP-1RA liraglutide would directly attenuate TX-induced platelet activation independent of weight loss or changes in glycemic control.

Prior efforts to establish platelet GLP-1R expression have been limited by nonspecific GLP-1R antibodies and probe-induced receptor activation. The Vanderbilt Institutional Review Board approved the following human subjects' research including the clinical trial registered on clinicaltrials.gov (Cardiovascular Effects of GLP-1 Receptor Activation; NCT03101930) from which samples were collected. To establish platelet GLP-1R expression, whole blood from healthy donors was treated with a proprietary platelet stabilizing cocktail (Patent #US010166248B1) to inhibit ex vivo platelet activation and stained with CD41, CD45, and a far-red fluorescent GLP-1R antagonist peptide (LUXendin645)[4] for flow cytometry. $CD45^{-}CD41^{+}$ platelets demonstrated higher expression of GLP-1R than $CD45^{+}CD41^{+}$ platelet-leukocyte aggregates and $CD45^{+}CD41^{-}$ leukocytes do (**Figure 1A**, left and middle). In a competitive inhibition assay, the selective GLP-1R antagonist exendin-(9-39) (EX9) reduced LUXendin645 staining on platelets in a dose-dependent manner at concentrations consistent with EX9 competition at GLP-1R (**Figure 1A**, right).[4]

To establish that GLP-1RAs regulate platelet function through the GLP-1R, we isolated platelet-rich plasma (PRP) from whole blood of adults with obesity and prediabetes defined by American Diabetic Association criteria (age 39.2 ± 14.2 years, 77.8% were female persons, body mass index 42.04 ± 6.11 kg/m^2, A_{1c} 5.7% ± 0.4%, n = 9). PRP adjusted to 250,000 platelets/μL was stimulated with TX receptor agonist, U44619, at a dose sufficient to elicit ≥60% platelet aggregation in the presence of liraglutide and EX9 vehicle. The effect of liraglutide or liraglutide plus EX9 antagonist on platelet aggregation was then assessed. TX-induced platelet aggregation with and without exposure to liraglutide were compared using a paired Student's *t*-test. In vitro exposure to liraglutide attenuated TX-induced platelet aggregation and the liraglutide effect was reversed by pretreatment with EX9 (**Figure 1B**, left) supporting the GLP-1R-dependent action of liraglutide and confirming GLP-1R function on platelets. Liraglutide attenuation of TX-induced platelet activation was heterogenous at baseline, which may result from common receptor allelic variants,[5] disease state, or medication use.[2]

To evaluate the in vivo effect of GLP-1RAs, PRP isolated from obese adults with prediabetes (age 40.3 ± 15.08 years, 60% were women, body mass index 43.05 ± 5.02 kg/m^2, A_{1c} 5.7% ± 0.3%) randomization 2:1:1 to liraglutide (0.6 mg/d for 1 week, 1.2 mg/d for 1 week, then 1.8 mg/d subcutaneously, n = 5), the dipeptidyl peptidase-4 inhibitor sitagliptin 100 mg/d (n = 2), or caloric restriction (390 kcal/d, n = 3) was stimulated

FIGURE 1 The GLP-1R on Platelets Attenuate Thromboxane-Induced Platelet Aggregation In Vitro and In Vivo



In healthy subjects, whole blood was treated with a platelet-stabilizing reagent and stained with CD41, CD45, and a far-red fluorescent glucagon-like peptide-1 receptor (GLP-1R) antagonistic peptide label (LUXendin645)[4] (50 nmol/L [nM]) for flowcytometry. **(A, left)** Representative gating strategy for $CD45^{-}CD41^{+}$ platelets, $CD45^{+}CD41^{+}$ platelet-leukocyte aggregates, and $CD45^{+}CD41^{-}$ leukocytes. **(Middle)** $CD45^{-}CD41^{+}$ platelets **(red line)** exhibit greater GLP-1R expression as compared to leukocytes **(orange line),** platelet-leukocyte aggregates **(blue line),** and the LUXendin645 (LUX) fluorescence minus one (FMO). **(Right)** In a competitive inhibition assay, pretreatment with the selective GLP-1R antagonist exendin-9-39 (EX9) for 5 minutes prior to LUXendin645 incubation **(blue and green lines)** attenuated LUXendin645 signal **(red line)** in a dose-dependent manner. (**B, left)** In subjects who are obese and prediabetic, PRP was pretreated with liraglutide (Lira) (100 nM) or vehicle for 30 minutes and then stimulated with U46619 ($n = 9$; $^{*}P < 0.05$, paired Student's *t*-test reported). In a representative subject, the effect of pretreatment with the selective EX9 (1 µM) for 5 minutes before liraglutide exposure is shown. **(Right)** Platelet aggregation at baseline and following intervention in a subset of subjects who are obese and prediabetic and randomized to treatment with liraglutide, sitagliptin, or dietary intervention was assessed. PRP was stimulated with equivalent doses of U44619, at baseline (BL) **(filled symbols)** and 2 weeks **(open symbols)** after randomization for each study participant based on the dose of U46619 required to elicit 60% or more platelet aggregation at baseline. $^{*}P < 0.05$, paired Student's *t*-test reported; $^{**}P < 0.01$ logistic regression reported for effect of liraglutide versus comparator treatments.

with U44619 to elicit ≥60% platelet aggregation at baseline and again after 2 weeks on intervention with the same dose of U46619. Two weeks of in vivo exposure to liraglutide significantly reduced TX-induced platelet aggregation from baseline (Figure 1B, right). After adjustment for baseline platelet responsiveness to TX, exposure to liraglutide reduced TX-induced platelet aggregation by 54% (95% CI: 18%-90%; $P = 0.003$, linear regression) compared to comparator treatments.

These are the first controlled human data to demonstrate that liraglutide attenuates TX-mediated platelet aggregation at low doses after short in vivo exposure. This inhibitory effect on the platelet occurred before liraglutide-induced weight loss (−0.12 ± 0.8 kg; $P = 0.89$). Platelet-specific GLP-1R expression and function were confirmed with the selective GLP-1R antagonist EX9, supporting a GLP-1R-dependent action of GLP-1RAs on platelets. Although the sample size is modest, these data support a unique and direct effect of a GLP-1RA on platelets not observed after 2 weeks of caloric restriction or dipeptidyl peptidase-4 inhibitor use. Longer exposure to liraglutide in vitro and selection of TX may explain why the GLP-1R-dependent actions of GLP-1RAs have not been previously reported.[2] Previous work demonstrated in vivo exposure to the DDP-4 inhibitor sitagliptin in subjects with T2DM for 1 month or 3 months attenuated thrombin-induced platelet aggregation (10% ± 2% and 30% ± 5%, respectively).[2] Our small sample size, short duration of exposure, non-T2DM study population, and use of a different platelet activator may account

for a lack of observed DDP-4 inhibitor effect on TX-induced platelet activation. TX-mediated platelet activation heightens both thrombosis and platelet-mediated inflammation.[3] Thus, the GLP-1R may represent a druggable platelet inhibitory receptor relevant in thrombotic and platelet-mediated inflammation disease. GLP-1RA-mediated attenuation of platelet activation tone may contribute to the clinical benefit observed in cardiovascular outcomes in T2DM. The low doses and short duration of exposure needed to attenuate platelet aggregation in prediabetic adults supports a weight loss–independent clinical benefit from GLP-1RAs in cardiovascular disease. This work points toward an expansion of the clinical populations that might benefit from treatment. Further study of the relationship between early attenuation of TX-mediated platelet activation following GLP-1RA exposure and thrombotic clinical outcomes are warranted.

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https://doi.org/10.1016/j.jacbts.2022.04.004

This work was supported by grants from the American Heart Association (17SFRN33520017); the Department of Medicine, Vanderbilt University Medical Center; and the National Institutes of Health (U19AI095227 and UL1 TR002243). Novo Nordisk and Merck provided investigational products. Dr Hodson was supported by Medical Research Council (MR/N00275X/1 and MR/S025618/1) and Diabetes UK (17/0005681) project grants. This project has received funding from the European Research Council under the European Union's Horizon 2020 Research and Innovation Programme (Starting Grant 715884 to Dr Hodson). Drs Hodson and Broichhagen have a licensing deal with Celtarys Research for distribution of LUXendin. Dr Beckman is a site-principal investigator in a Novo Nordisk–sponsored clinical trial in peripheral arterial disease. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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