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Atherogenic lipid stress induces platelet hyperactivity through CD36-mediated hyposensitivity to prostacyclin: the role of phosphodiesterase 3A

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ABSTRACT

Prostacyclin (PGI_2) controls platelet activation and thrombosis through a cyclic adenosine monophosphate (cAMP) signaling cascade. However, in patients with cardiovascular diseases this protective mechanism fails for reasons that are unclear. Using both pharmacological and genetic approaches we describe a mechanism by which oxidized low density lipoproteins (oxLDL) associated with dyslipidemia promote platelet activation through impaired PGI_2 sensitivity and diminished cAMP signaling. In functional assays using human platelets, oxLDL modulated the inhibitory effects of PGI_2, but not a phosphodiesterase (PDE)-insensitive cAMP analog, on platelet aggregation, granule secretion and *in vitro* thrombosis. Examination of the mechanism revealed that oxLDL promoted the hydrolysis of cAMP through the phosphorylation and activation of PDE3A, leading to diminished cAMP signaling. PDE3A activation by oxLDL required Src family kinases, Syk and protein kinase C. The effects of oxLDL on platelet function and cAMP signaling were blocked by pharmacological inhibition of CD36, mimicked by CD36-specific oxidized phospholipids and ablated in $CD36^{-/-}$ murine platelets. The injection of oxLDL into wild-type mice strongly promoted FeCl3-induced carotid thrombosis *in vivo*, which was prevented by pharmacological inhibition of PDE3A. Furthermore, blood from dyslipidemic mice was associated with increased oxidative lipid stress, reduced platelet sensitivity to PGI_2 *ex vivo* and diminished PKA signaling. In contrast, platelet sensitivity to a PDE-resistant cAMP analog remained normal. Genetic deletion of CD36 protected dyslipidemic animals from PGI_2 hyposensitivity and restored PKA signaling. These data suggest that CD36 can translate atherogenic lipid stress into platelet hyperactivity through modulation of inhibitory cAMP signaling.

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Introduction

Myocardial infarction (MI) is characterized by platelet-driven atherothrombotic events that lead to acute occlusion of a coronary vessel. Excessive platelet activation is controlled by endothelial derived nitric oxide (NO) and prostacyclin (PGI_2),[1] but action of these protective agents is overcome in MI by mechanisms that are yet to be elucidated. A key risk factor for MI is dyslipidemia, which is strongly associated with a pro-thrombotic phenotype linked to atherothrombosis and platelet hyperactivity.[2,3] The blood of high-risk individuals with dyslipidemia is characterized by increased plasma lipid peroxides, with low density lipoproteins (LDL) serving as a highly abundant carriers for these oxidatively-modified lipids.[4-6] Oxidized LDL (oxLDL) are circulating pathological ligands that can enhance thrombosis through

their ability to promote platelet hyperactivity. *In vitro* experimentation demonstrates that these modified lipoproteins can cause direct activation of platelets and also potentiate platelet activation induced by physiological agonists such as thrombin, ADP and epinephrine.[7–10] However, the potential pathophysiological importance of these observations for thrombosis in vivo remain unclear.

The scavenger receptor CD36 has emerged as a potential conduit for transducing plasma lipid stress into platelet hyperactivity and thrombosis, through the recognition of lipoprotein associated molecular patterns (LAMPs). CD36, alone or potentially in combination with Toll-Like Receptor (TLR)[2] and TLR6 drive a complex series of intracellular signaling events that are associated with platelet activation.[11–15] Upon ligation of CD36, Src family kinases constitutively associated with the receptor, drive the activation of Syk, Vav-1, PLCγ2, ERK5 and JNK that are associated with platelet activation.[13,16–18] More recently, data have emerged to suggest that the signaling events promote the generation of reactive oxygen species (ROS).[14,16,17] ROS in turn activate ERK to drive thrombosis directly by platelet hyperactivity and caspase-dependent procoagulant activity.[18,19] Moreover, we found that ROS diminish sensitivity to the nitric oxide (NO)-stimulated cGMP-PKG inhibitory signaling cascade to reduce the threshold for platelet activation.[17] These data suggest that the translation of atherogenic lipid stress by platelet CD36 is functionally linked to both stimulation of activatory signaling pathways and to an as of yet ill-defined modulation of inhibitory pathways.

PGI_2 is the most potent endogenous regulator of platelet function with both genetic and pharmacological modulation of the pathway linked to accelerated thrombosis *in vivo*.[20] PGI_2 activates a cyclic adenosine monophosphate (cAMP) signaling pathway that leads to subsequent activation of protein kinase A (PKA) in platelets and results in the phosphorylation of numerous proteins,[21] linked to the inhibition of $Ca2^+$ mobilization, dense granule secretion, spreading, integrin $\alpha_{IIb}\beta_3$ activation and aggregation *in vitro*.[20] To ensure optimal platelet function, cAMP levels are tightly regulated by the hydrolyzing enzymes phosphodiesterase (PDE)2A and 3A. The pharmacological inhibition or genetic ablation of PDE3A in murine and human platelets reduces thrombotic potential.[22,23] Thus, factors that alter platelet inhibition by influencing cAMP synthesis or hydrolysis may be critical modulators of atherothrombosis and potentially lead to a pro-thrombotic phenotype. Given the established link between oxidized lipid stress and excessive platelet activation, the aim of this study was to determine if oxidatively modified lipoproteins could promote platelet hyperactivity through modulation of the PGI_2/cAMP signaling pathway.

Methods

Reagents

Phospho-PKA Substrate (RRXS*/T*) Rabbit mAb and phospho-VASP-Ser$^{157/239}$ antibodies were from Cell Signaling Technology (Danvers, USA). PDE3A antibodies were from the MRC Unit (Dundee University, Dundee, UK). Anti-β-Tubulin antibody was from Millipore (Nottingham, UK). BD Phosflow Lyse/Fix Buffer was from BD Biosciences (Oxford, UK). OxPC-E06 mAb was from Avanti Polar Lipids (Alabaster, USA). FITC-labeled Rat Anti-Mouse P-selectin (CD62P) and PE-labeled JON/A antibodies were from Emfret Analytics (Würzburg, Germany). Alexa-Fluor 647 Goat anti-Rabbit IgG, Alexa-Fluor 488 Succinimidyl Ester and Pacific Blue Succinimidyl Ester were from ThermoFisher Scientific (Waltham, USA). PAR-1 peptide was from Anaspec (Fremont, USA). Anti-CD36 Antibody (FA6-152) was from Abcam (Cambridge, UK). Phosphodiesterase Activity Assay Kit was from Enzo Life Sciences (Exeter, UK). cAMP Biotrack EIA was from GE Healthcare (Buckinghamshire, UK). Horm Collagen was from Nycomed (Munich, Germany). PGI_2 and Cholesterol Assay Kit were from Cayman Chemical (Cambridge, UK). Vena8 Endothelial+ biochips were from Cellix (Hertfordshire, UK). All other reagents were from Sigma-Aldrich (Dorset, UK).

Experimental animals

CD36$^{-/-}$ mice were provided by Prof. Maria Febbraio (University of Alberta, Canada). C57BL/6 were from Charles River (Kent, UK). For high-fat diet studies, male mice were fed a 45% Western diet (Special Diet Services) for 12–16 weeks. Sex/age-matched littermates were fed a normal chow for the duration of the experiments and used as controls. For all remaining experiments, male C57BL/6 and CD36-/- were used at eight weeks of age.

Isolation and oxidation of plasma low density lipoproteins

Low-density lipoproteins (density 1.019–1.063 g/mL) were prepared from fresh human plasma by sequential density ultracentrifugation and oxidised with $CuSO_4$ (10 μmol/L).[14] Separate preparations of LDL were used to repeat the individual experiments.

Platelet aggregation, flow assays, flow cytometric analysis, intravital microscopy, immunoprecipitation, immunoblotting, phosphodiesterase enzyme activity assay and cyclic adenosine monophosphate measurement

Detailed methods are presented in the *Online Supplementary Methods*.

Statistical analysis

Experimental data was analyzed by Graphpad Prism 6 (La Jolla, CA, USA). Data are presented as means±standard error of the mean (SEM) of at least three different experiments. Differences between groups were calculated using Mann-Whitney U Test or Kruskal-Wallis Test for non-parametric testing and statistical significance accepted at $P \leq 0.05$.

All studies were approved by the Hull York Medical school Ethics committee and University of Leeds Research Ethics committee.

Results

Oxidized low density lipoproteins cause platelet hyposensitivity to prostacyclin

Treatment of human washed platelets with PGI_2 (20 nM) for one minute (min), at a time point that induces maximal cAMP-mediated signaling[24] reduced thrombin (0.05 U/mL)-induced aggregation from 89.0±4.1 to 9.4±4.4% ($P<0.01$) (Figure 1A). Next, platelets were treated with oxLDL or control native LDL (nLDL) (50 μg/mL) for 2 min prior to the addition of PGI_2 (20 nM) and thrombin (0.05 U/mL). The presence of oxLDL caused a partial recovery in thrombin-stimulated platelet aggregation to 50.0±9.3 ($P<0.015$ *vs.* control), without stimulating aggregation directly (Figure 1A). In contrast, PGI_2-mediated

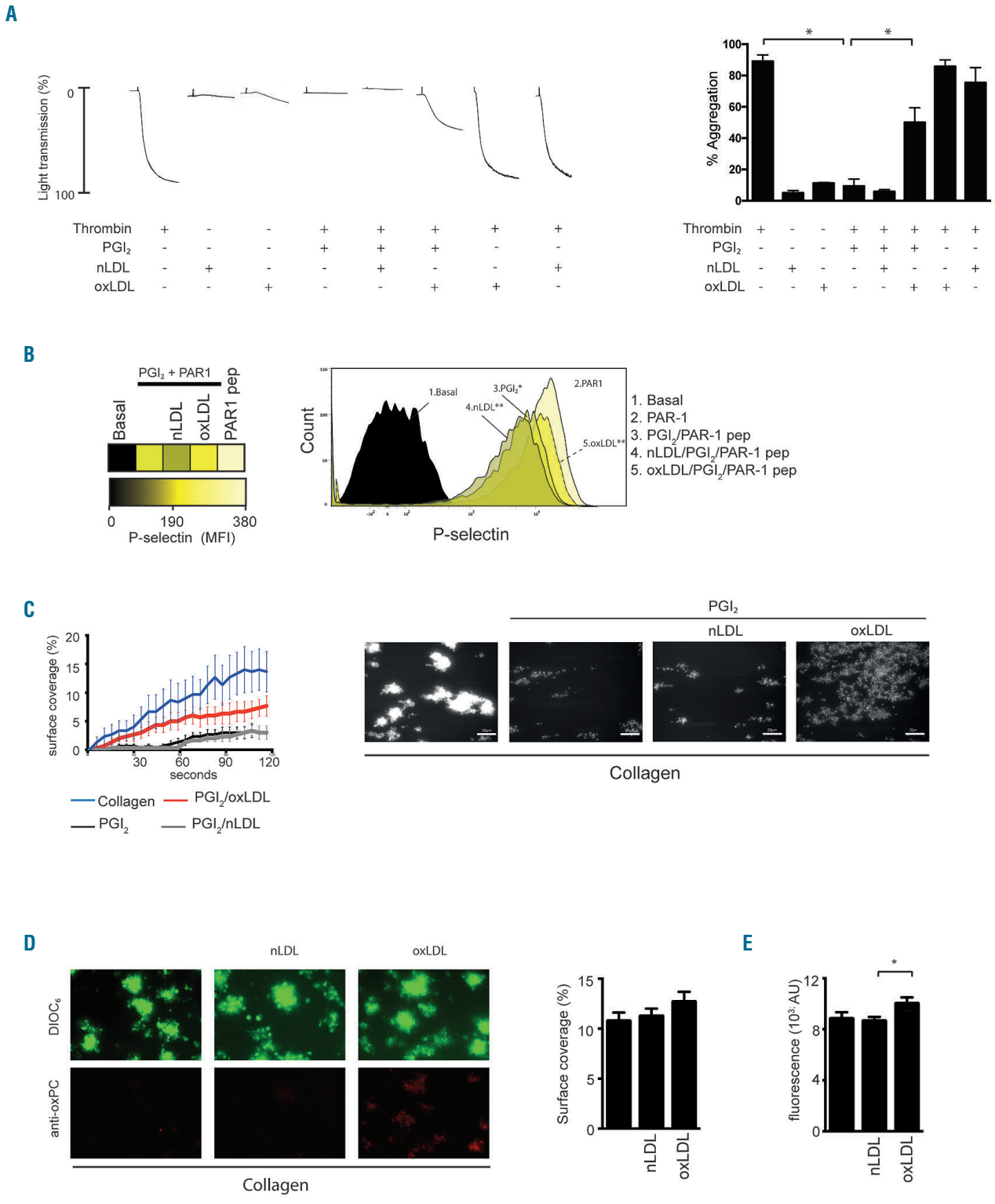

Figure 1. Oxidized low-density lipoproteins (oxLDL) induce prostacyclin (PGI_2) hyposensitivity in platelets. (A) Washed human platelets (2.5 x 10^8/mL) were treated alone or with control native LDL (nLDL) or oxLDL (50 μg/mL) for 2 minutes (min) followed by a 1 min incubation with PGI_2 (20 nM). Thrombin (0.05U/mL)-stimulated aggregation was then measured under constant stirring (1000 rpm) at 37°C for 4 min. In some cases, platelets were incubated with either thrombin, nLDL or oxLDL alone for 4 min. (Left) Representative aggregation traces. (Right) Percentage aggregation is presented by mean±standard error of mean (SEM) (n= 5). ***P*<0.01 compared to platelets treated with thrombin and PGI_2, Mann-Whitney U Test. (B) Whole blood was treated as in (A) and activated with PAR-1 peptide (10 ∝M) for 5 min followed by fixation. CD62P expression was assessed by flow cytometry. Representative data of three independent experiments. (Left) Data are presented as heatmap of mean fluorescence intensity (MFI). (Right) Representative histograms. (C) Human whole blood was incubated with PGI_2 (20 nM) for 1 min alone or with control native LDL (nLDL) or oxLDL (50 μg/mL) for 10 min. Blood was perfused over collagen-coated biochips for 2 min at arterial shear 1000s-1 and images of adherent platelets were taken by fluorescence microscopy. (Left) The surface coverage (%) is presented as mean±SEM (n=3) (*P*<0.05, Mann-Whitney U Test). (Right) Representative fluorescence microscopy images are shown. (D) Whole blood was incubated with nLDL or oxLDL (50 μg/mL) for 10 min and then perfused over collagen-coated surfaces for 2 min at arterial shear 1000s-1. Thrombi were post-stained with anti-oxPC antibody and images were taken by fluorescence microscopy. (Left) Representative of images of 3 independent experiments. Stained with DIOC6 (top panel) or anti-oxPC (bottom panel) (Center) The surface coverage is presented as mean±SEM (n=4, **P*<0.05, Mann-Whitney U Test). (Right) Fluorescence (red Channel) is presented as mean±SEM (n=4, **P*<0.05, Mann-Whitney U Test).

inhibition was unaffected by nLDL (5.8±1.2%). Similar data were obtained when platelets were stimulated with collagen (*Online Supplementary Figure S1)*. Next, the effects of oxLDL in whole blood were examined. Stimulation of whole blood with PAR-1 peptide (10 μM) increased *P*-selectin expression, which was reduced by pre-treatment with PGI_2 (20 nM). Consistent with the aggregation experiments, oxLDL reduced inhibitory effects of PGI_2 (Figure 1B). Finally, the effects of oxLDL under physiological conditions of arterial flow were examined. Perfusion of whole blood over collagen-coated biochips led to platelet deposition and thrombus formation (Figure 1C), with surface coverage inhibited by PGI_2 (20 nM) from 14.9±2.9 to 3.8±1.7% (P<0.05). OxLDL, but not nLDL, prevented PGI_2-mediated inhibition of platelet deposition (8.1±1.9%; P<0.05 compared to PGI_2 alone). OxLDL alone caused a small but non-significant increase in thrombosis and was incorporated into the thrombi as evidenced by staining for oxidized lipid epitopes (Figure 1D). These data demonstrate that under a variety of different conditions oxLDL reduces platelet sensitivity to PGI_2.

Oxidized low density lipoproteins modulate cyclic adenosine monophosphate signaling through increased phosphodiesterase 3A activity

PGI_2 inhibits platelets through the stimulation of cAMP-PKA signaling cascade.[20] Given the reduced platelet sensitivity to PGI_2, the direct effect of oxLDL on platelet cAMP metabolism was tested. Incubation with PGI_2 caused a significant increase in platelet cAMP concentrations (1814±166 fmol cAMP/1x10^8 platelets; P<0.05 *vs.* basal). When platelets were treated with nLDL (50 μg/mL), the ability of the prostanoid to elevate cAMP was unaffected (1885±203 fmol cAMP/1x10^8 platelets), while oxLDL (50 μg/mL) prevented PGI_2-induced accumulation of cAMP (481±23 fmol cAMP/1x10^8 platelets; P<0.05 compared to PGI_2 alone) and also reduced basal cAMP concentrations (not significant) (Figure 2Ai). To determine if oxLDL blocked cAMP synthesis or accelerated cAMP breakdown by phosphodiesterase 3A (PDE3A) and PDE225,[25] the PDE2A inhibitor EHNA (20 μM) and the PDE3A inhibitor milrinone (10 μM) were used. Consistent with previous studies, both inhibitors potentiated cAMP

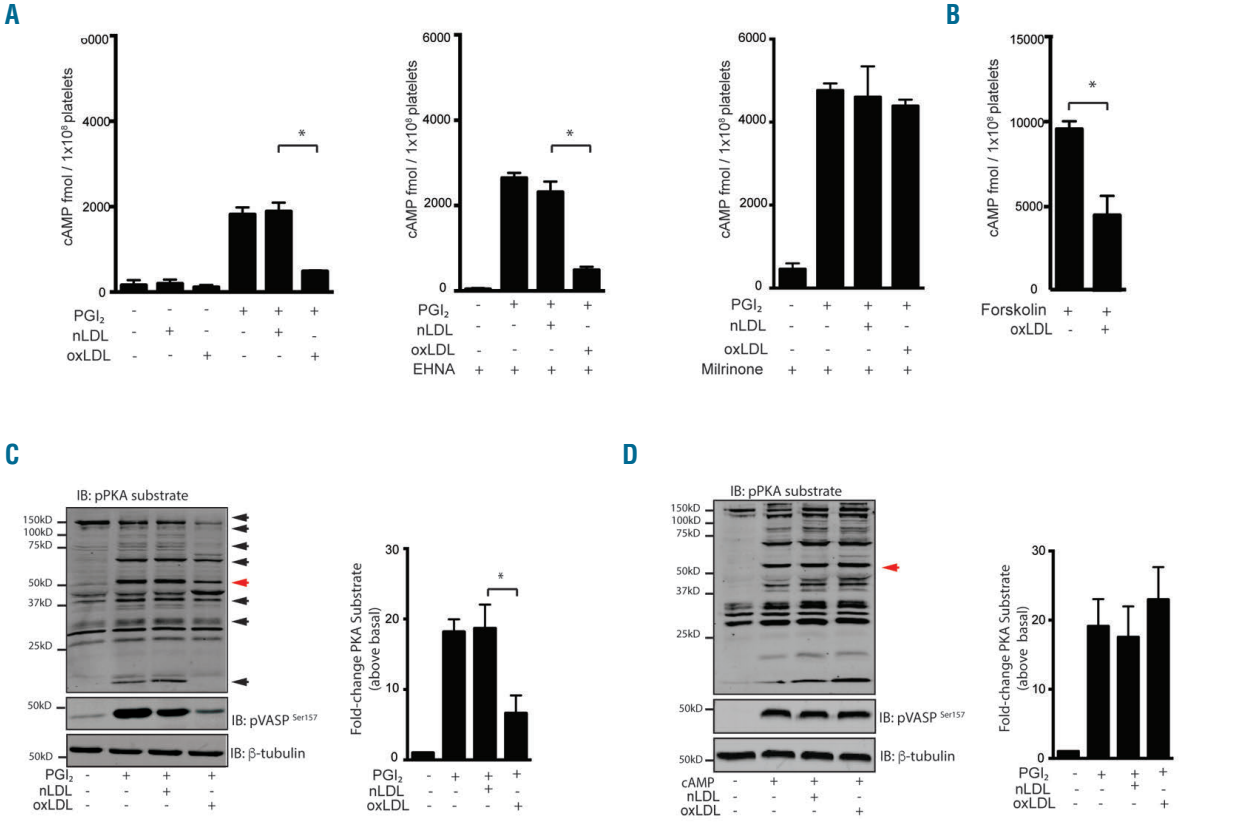

Figure 2. Oxidized low-density lipoproteins modulate cyclic adenosine monophosphate (cAMP) signaling in response to prostacyclin (PGI_2). (A) Washed human platelets (2x10^8/mL) incubated with apyrase, indomethacin and EGTA were treated alone or with control native LDL (nLDL) or oxLDL (50 μg/mL) for 2 minutes (min) followed by a 1 min PGI_2 (50 nM) incubation. Platelets were lysed and intracellular cAMP concentrations were measured by enzyme immunoassay. (Left) Intracellular cAMP levels presented as mean±standard error of mean (SEM) (n=3, *P<0.05, Mann-Whitney U Test). (Center) Platelets treated as described under (A) in the presence EHNA (20 μM). Intracellular cAMP levels are presented as mean±SEM (n=3, *P<0.05, Mann-Whitney U Test) (Right) Platelets treated as described under (A) in the presence of Milrinone (10 μM). Intracellular cAMP levels are presented as mean±SEM (n=3, *P<0.05, Mann-Whitney U Test). (B) Washed human platelets (2x10^8/mL) were incubated alone or with oxLDL (50 μg/mL) for 2 min followed by a 5-min incubation with Forskolin (10 μM), then lysed and measured as described under (A). Intracellular cAMP concentrations presented as mean±SEM (n=3, *P<0.05, Mann-Whitney U Test). (C) Washed human platelets (5x10^8/mL) were treated as described under (A), lysed in Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-phosphoPKA substrate, anti-phosphoVASPser^{157} and anti-β tubulin. (Left) Representative blot of three independent experiments. (Right) Densitometry of the representative highlighted band (red) (*P<0.05, Mann-Whitney U Test). (D) Washed human platelets were treated as described under (A) except that platelets were treated with 8-CPT-cAMP (50 μM) for 5 min and then processed as in (C). (Left) Representative blot of three independent experiments and (Righti) densitometry of the representative highlighted band (red).

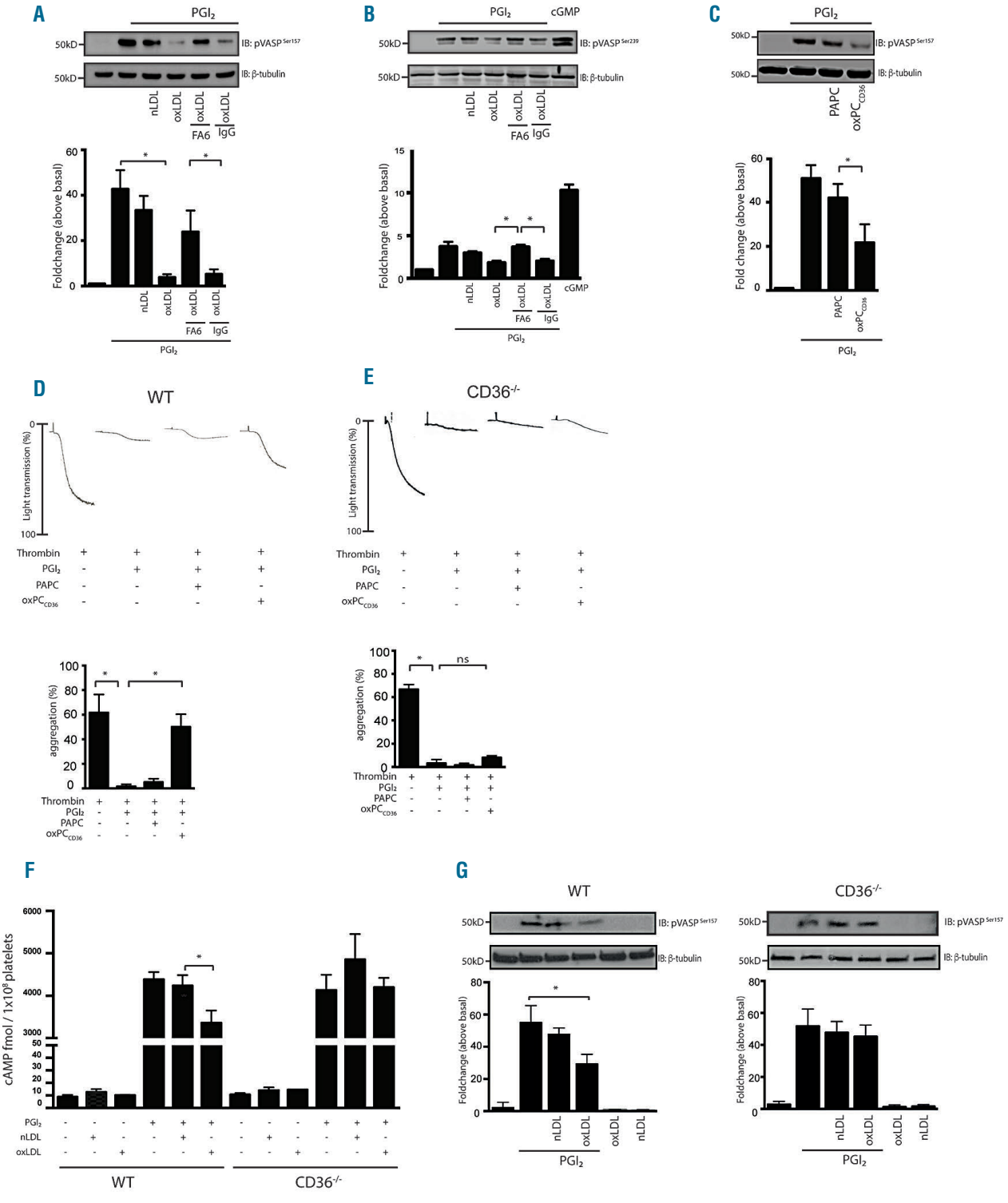

Figure 3. Oxidized low-density lipoproteins (oxLDL) and oxPCCD36 alter prostacyclin (PGI_2) inhibitory signaling *via* CD36. (A) Washed human platelets ($5x10^8$/mL) incubated with apyrase, indomethacin and EGTA were incubated with FA6-152 or IgG (1 μg/mL) for 20 minutes (min). Platelets were then incubated alone or with control native LDL (nLDL) or oxLDL (50 μg/mL) for 2 min and subsequently stimulated by PGI_2 (50nM) for 1 min. Treated platelets were lysed in Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-phosphoVASPser^{157} or anti-β tubulin. (Top) Representative blot of three independent experiments. (Bottom) Densitometry of pVASPser^{157} fold-change above basal mean±standard error of mean (SEM) (n=3 *P<0.05, Mann-Whitney U Test). (B) As in (A) except that platelets were probed for pVASPser^{239}. (C) Platelets were treated as in (A) alone or with oxPCCD36 or PAPC (25 μM) for 2 min followed by PGI_2 (50 nM) for 1 min. (Top) Representative blot of three independent experiments and (Bottom) Densitometry of pVASPser157 fold-change above basal, mean±SEM (n=3 *P<0.05, Mann-Whitney U Test). (D) Washed wild-type murine platelets ($2.5x10^8$/mL) were treated alone or with oxPCCD36 or PAPC (10 μM) for 2 min followed by a 1 min incubation with PGI_2 (20 nM). Thrombin (0.05 U/mL)-stimulated aggregation was then measured under constant stirring (1000 rpm) at 37 °C for 4 min. (Top) Representative aggregation traces and (Bottom) data presented as percentage aggregation, mean±SEM (n=3, P<0.05, Mann-Whitney U Test). (E) As described in (D) for CD36$^{-/-}$ platelets. Ns: not significant. (F) Washed murine WT or CD36$^{-/-}$ platelets ($2x10^8$/ml) were treated alone or with nLDL or oxLDL (50 μg/mL) for 2 min followed by a 1-min PGI_2 (50 nM) incubation. Platelets were lysed, and intracellular cAMP concentrations were measured by enzyme immunoassay. Intracellular cAMP levels are presented as mean±SEM (n=3, *P<0.05, Mann-Whitney U Test). (G) As in (A) except that murine (Left) WT and (Right) CD36-/- platelets were used. Representative blot of three independent experiments. pVASP^{ser157} is presented as beta-tubulin corrected fold-change above basal±SEM (n=3 *P<0.05, Mann-Whitney U Test).

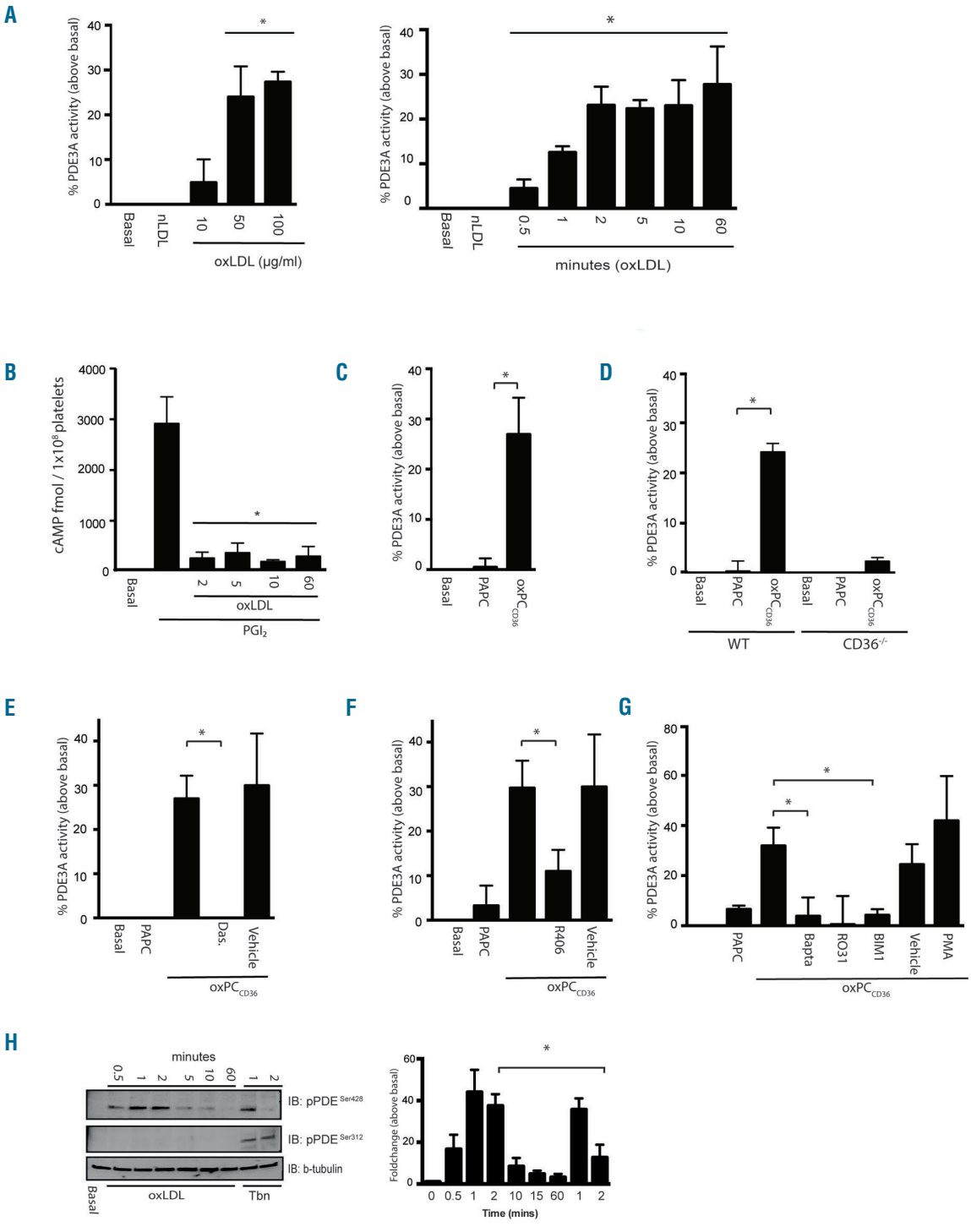

Figure 4. Oxidized low-density lipoproteins (oxLDL) and oxPCCD36 induce sustained phosphodiesterase 3A (PDE3A) activity in a CD36-dependent manner. (A). Washed human platelets (5x10^8/mL) incubated with apyrase, indomethacin and EGTA were (Left) incubated with nLDL (50 μg/mL) or oxLDL (10-100 μg/mL) for 2 minutes (min) or (Right) incubated with control native LDL (nLDL) (50 μg/mL) or oxLDL (50 μg/mL) for up to 60 min. Platelets were lysed, PDE3A was immunoprecipitated and enzyme activity was measured. Data is expressed as % activity above basal activity and presented as mean±standard error of mean (SEM) (P<0.05; n=3, Kruskal-Wallis Test). (B) Washed human platelets (5x10^8/mL) were incubated with oxLDL (50 μg/mL) for up to 60 min followed by PGI_2 (50 nM) for 1 min. Platelets were lysed, and intracellular cyclic adenosine monophosphate (cAMP) levels were determined by enzyme immunoassay. Data are presented as mean±SEM (*P<0.05, n=3, Kruskal-Wallis Test). (C) Washed human platelets (5x10^8/mL) were incubated with oxPCCD36 or PAPC (25 μM) for 2 min, and activity of immunoprecipitated PDE3A measured. Data are expressed as % activity above basal activity and presented as mean±SEM (P<0.05; n=3 Mann-Whitney U Test). (D) As in (C), except that PDE3A activity was measured in wild-type (WT) and CD36$^{-/-}$ washed platelets (*P<0.05; n=3 Mann-Whitney U Test). (E) As in (A) except that human platelets (5x10^8/mL) incubated with apyrase, indomethacin and EGTA were then treated with oxPCCD36 or PAPC (25 μM) for 2 min in the presence of absence of dasatinib (10 μM) or vehicle control (DMSO). PDE3A was immunoprecipitated, and enzyme activity was measured. Data are expressed as % activity above basal activity and presented as mean±SEM (P<0.05; n=4 Mann-Whitney U Test). (F) As in (A) except that platelets were treated with R406 (1 μM) or vehicle control (DMSO). (G) As in (A), except that platelets were treated with PKC inhibitors (RO31 10μM, BIM1 10μM), PMA (100nM), BAPTA (20 μM) or vehicle control (DMSO). (n=3, *P<0.05 compared to basal, Kruskal-Wallis Test). (H) Washed human platelets (5x10^8/mL) treated with apyrase, indomethacin and EGTA were incubated with or oxLDL (50 μg/mL; 0-60 min) or thrombin (0.1 U/mL; 0-2min). Treated platelets were lysed in Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-phosphoPDE3Aser^{312}, phosphoPDE3Aser^{428} or anti-β tubulin. (Left) Representative blot of three independent experiments. (Right) Densitometry for phosphoPDE3Aser^{428} is presented as fold-change above basal, mean±SEM (n=3, *P<0.05, Kruskal-Wallis Test).

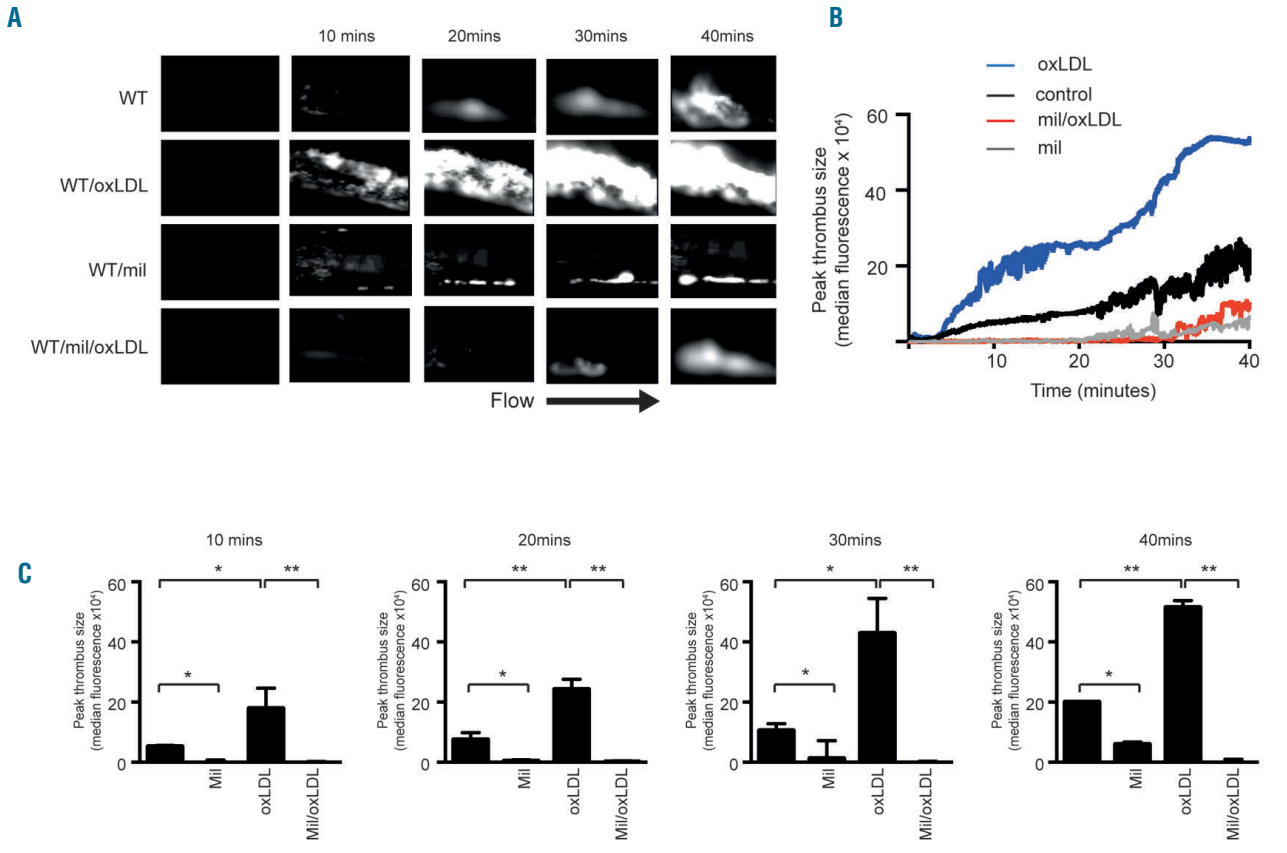

Figure 5. Oxidized low-density lipoproteins induced thrombosis *in vivo* is prevent by inhibition of phosphodiesterase 3A (PDE3A). Intravital microscopy was performed as described in the *Online Supplementary Methods*. (A) Representative fluorescence images of thrombi formed under different conditions are shown over the course of 40 minutes (min) after vascular injury. Black arrow shows the direction of blood flow. (B) Representative median integrated fluorescence signals of Rhodamine G obtained from an individual carotid thrombus under different conditions. (C) Quantified median integrated fluorescence signals from 10, 20, 30 and 40 min after vascular injury taken from five wild-type (WT) mice for each treatment. *P<0.05; **P<0.01, Kruskal-Wallis Test.

accumulation in response to PGI_2 .[26,28] OxLDL reduced cAMP levels in the presence of EHNA (2645±122 to 488±7623 fmol cAMP/1x10^8 platelets; P<0.05), but failed to prevent cAMP accumulation in the presence of milrinone (4761±170 to 4386±15723 fmol cAMP/1x10^8 platelets; P<0.05) (Figure 2Aii and 2Aiii). To confirm that the reduction in cAMP accumulation was not restricted to PGI_2, platelets were stimulated with forskolin, which increases cAMP in a receptor-independent non-compartmentalized mechanism. Forskolin (10 μM)-stimulated elevation in cAMP was prevented by preincubation with oxLDL (9506±526 to 4506±1136 fmol cAMP/2x108 platelets; P<0.05) (Figure 2B).

Next, the effects of oxLDL on cAMP signaling were assessed. μ (50 nM) induced robust phosphorylation of a number of PKA substrates with apparent molecular weights of: 150, 100, 75, 70, 50, 37 and 20kDa (Figure 2C upper panel), and specifically vasodilator-stimulated phosphoprotein (VASP) (phosphoVASP-ser157) (Figure 2C middle panel) an established target of PKA signaling.27 These phosphorylation events were diminished by oxLDL (50 μg/mL), but unaffected by nLDL (50 μg/mL) (Figure 2Cii). To further establish that the reduced signaling response was due to enhanced cAMP hydrolysis, the experiment was repeated with 8-CPT-6-Phe-cAMP (50μM), a cell permeable non-hydrolysable (PDE resistant) analog of cAMP (*Online Supplementary Figure S2*). Using a concentration that produced an equivalent quantity of intracellular cAMP (*Online Supplementary Figure S3*) as μ (50 nM), 8-CPT-6-Phe-cAMP caused robust phosphorylation of PKA substrates, which were unaffected by oxLDL (Figure 2D). These data suggest that oxLDL may regulate platelet sensitivity to μ through modulation of the cAMP-signaling cascade by PDE3A.

CD36 is required for oxidized low-density lipoprotein modulation of cyclic adenosine monophosphate signaling

Previously, we and others have shown that CD36 transduces the effects of oxLDL into platelet hyperactivity.[14,18,28,29] To examine the role of CD36 in linking oxLDL to altered cAMP signaling, we used a three-pronged strategy: (i) the CD36-blocking antibody FA6-152; (ii) the oxidized phospholipid oxPCCD36, a CD36-specific pathological ligand present in oxLDL,[26] and (iii) murine platelets deficient in CD36 (*Online Supplementary Figure S4*). This strategy, particularly the use of oxPCCD36, was used to account for differences in human and murine platelet sensitivity to human oxLDL. PGI_2 induced a robust phosphorylation of both the preferred ser^{157} site and the alternative PKA phosphorylation site ser^{239} in human platelets,[30] which was reduced significantly by oxLDL. The presence

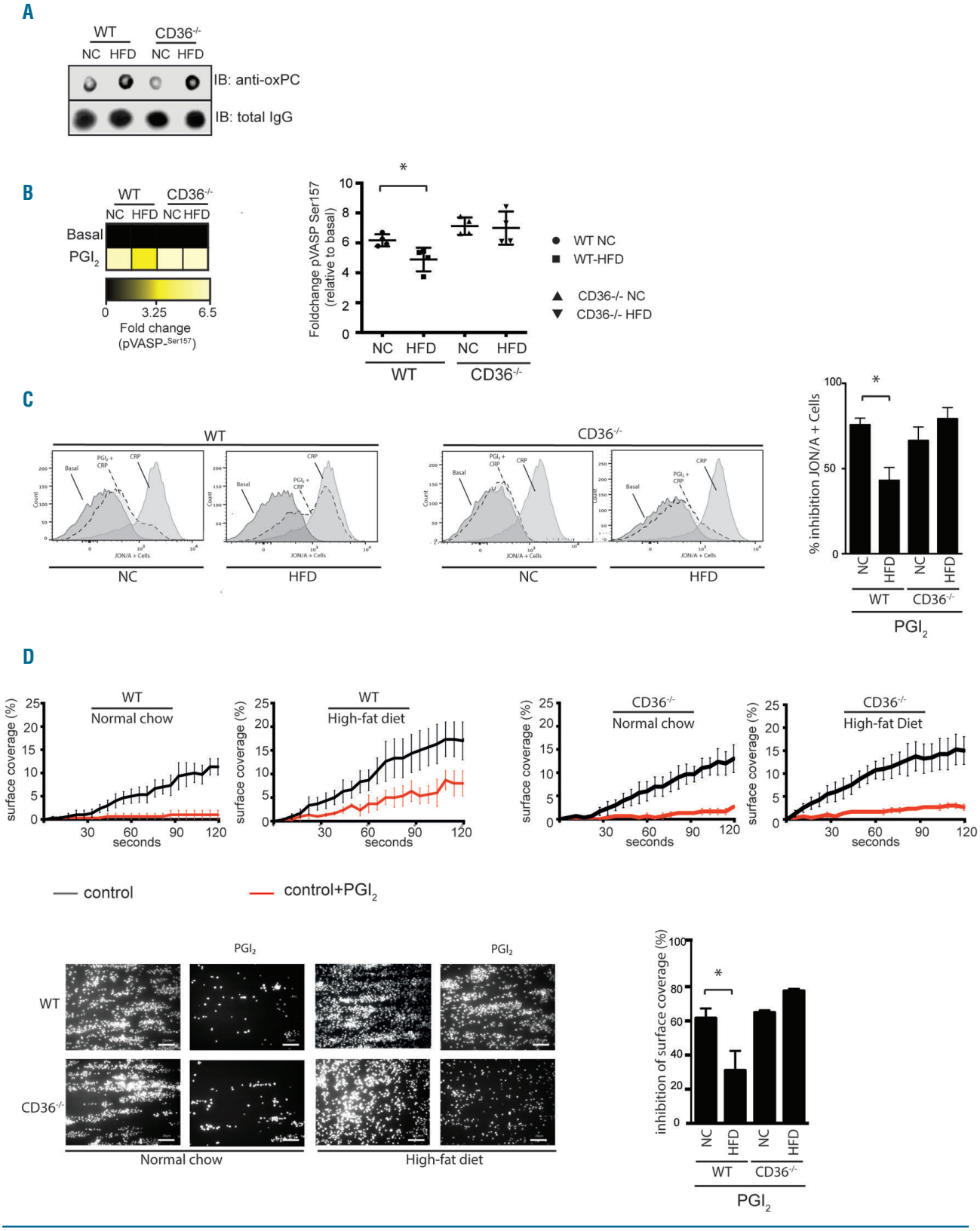

Figure 6. High-fat diet fed mice correlate with increased plasma levels of oxidized phospholipids to reduced prostacyclin (PGI_2) sensitivity *via* CD36. (A) Representative dot blots of mouse plasma probed with anti-oxidized phospholipids (HRP-conjugated-E06) and total-IgG (HRP-conjugated anti-mouse). (B) Whole blood from normal chow and high-fat fed animals were treated with PGI_2 (100nM) for 1 minute (min). Blood was fixed, permeabilized and incubated with anti-pVASPser[157] followed by secondary fluorescent-conjugate (Alexa 647) and analyzed by flow-cytometry. Representative heat map of fold increase in phosphoVASPser[157]. Quantification is presented as fold-change of median fluorescence intensity above basal (n=4, *P<0.05, Mann-Whitney U Test). (C) Whole blood from normal chow and high-fat diet fed animals were treated with PGI_2 (100 nM) for 1 min followed by CRP (10 μg/mL) for 5 min. Blood was fixed and JON/A positive cells were analyzed by flow cytometry. (Left) Representative histograms. (Right) Data presented as percentage inhibition of JON/A binding, mean±standard error of mean (SEM) (n=4 *P<0.05, Mann-Whitney U Test). (D) Whole blood incubated alone or with PGI_2 (20 nM) for 1 min was perfused at arterial shear 1000s^{-1} for 2 min over a collagen matrix (50 μg/mL). Images of adherent platelets were taken by fluorescence microscopy. (Top) surface coverage (%) presented as a function of time. (Bottom left) Representative images of arterial flow experiments, (Bottom right) Data presented as inhibition of surface coverage (%), mean±SEM (n=5; *P<0.05, Mann-Whitney U Test).

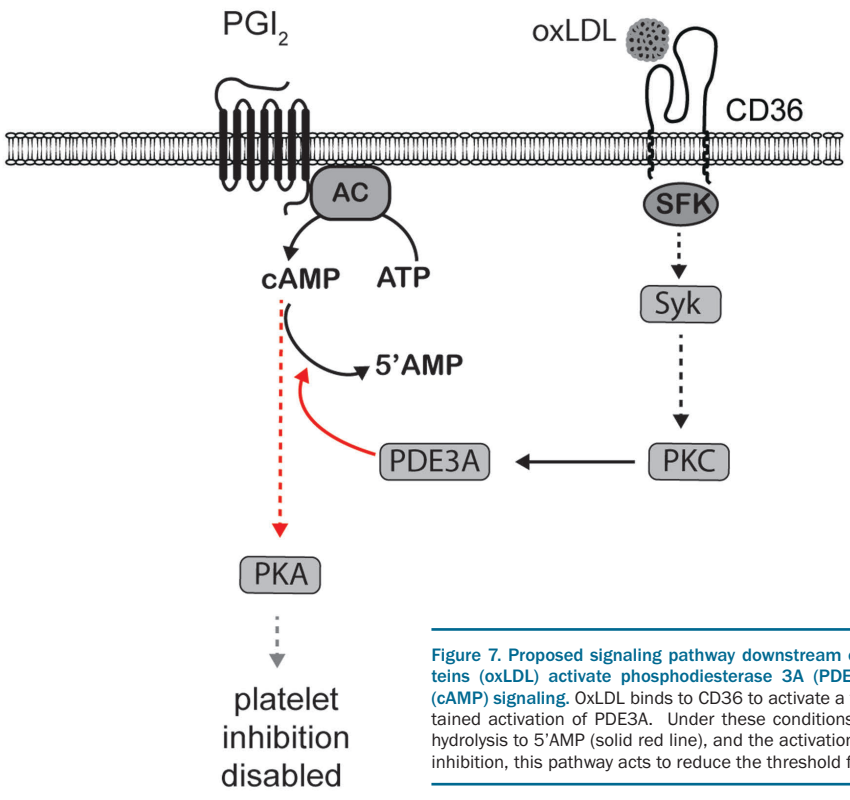

Figure 7. Proposed signaling pathway downstream of CD36 through which oxidized low-density lipoproteins (oxLDL) activate phosphodiesterase 3A (PDE3A) to suppress cyclic adenosine monophosphate (cAMP) signaling. OxLDL binds to CD36 to activate a tyrosine kinase signaling pathway that leads to a sustained activation of PDE3A. Under these conditions cAMP is maintained at low concentrations through hydrolysis to 5'AMP (solid red line), and the activation of PKA is diminished (dashed red line). Through disinhibition, this pathway acts to reduce the threshold for platelet activation and promote thrombosis.

of FA6-152 (1 μg/mL), but not control IgG (1 μg/mL), caused a significant recovery in phosphoVASP-ser157 (Figure 3A and B). OxPCCD36 (25 μM), but not the control lipid, PAPC (25 μM), diminished phospoVASP-ser157 in response to PGI_2 (Figure 3C). Thrombin-induced aggregation of wild-type (WT) and $CD36^{-/-}$ platelets was indistinguishable, and and PGI_2 (20 nM) caused complete inhibition of aggregation with only shape change remaining (Figure 3D and E). However, in CD36-deficient platelets, oxPCCD36 (25 μM) did not influence the inhibitory effects of PGI_2 (Figure 3E). To confirm the mechanism underpinning these observations, we examined cAMP signaling. PGI_2 (20 nM) caused a significant increase in cAMP in both WT and $CD36^{-/-}$ platelets (4382±175 and 4128±366 fmol cAMP/1x10^8 platelets). Preincubation with oxLDL, but not nLDL, reduced cAMP accumulation in WT mice (3347±294 fmol cAMP/1x10^8 platelets; $P<0.05$) but not $CD36^{-/-}$ (4196±224fmol cAMP/1x10^8 platelets) (Figure 3F). Furthermore, PGI_2-induced phosphoVASP-ser157 (Figure 3G) and ser239 (*Online Supplementary Figure S5*) were reduced by oxLDL in WT but not $CD36^{-/-}$ platelets.

Atherogenic lipid stress induces platelet phosphodiesterase 3A activity through a mechanism that requires CD36, Src kinases and protein kinase C

Our data suggested that ligation of CD36 could activate PDE3A. We tested this directly measuring enzymatic activity of PDE3A immunoprecipitated from platelets treated with either oxLDL or oxPCCD36. PDE activity in the immunoprecipitated samples was blocked by milrinone confirming enzyme activity was due to PDE3A (*Online Supplementary Figure S6*). OxLDL (10-100 μg/mL) caused a concentration-dependent increase in PDE3A activity (Figure 4A, left), which plateaued at 50 μg/mL (24±6.8%; $P<0.05$ compared to basal) and was maintained at 27.7%±8.5 above basal for up to 60 min (longest time tested) (Figure 4A, right). This was strikingly different from the physiological agonist thrombin which induced a rapid induction of PDE3A activity that peaked at 1 min before returning to basal after 5 min (*Online Supplementary Figure S7*).

To link the sustained activation of PDE3A to decreased cAMP levels, we assessed the cyclic nucleotide concentrations over time. Platelets were incubated with oxLDL for up to 60 min, followed by a 1 min treatment with PGI_2 (50 nM) before measuring cAMP concentrations. The preincubation of platelets with oxLDL, but not nLDL (*data not shown*), for up to 60 min significantly blunted PGI_2-induced increases in cAMP with concentrations remaining at basal levels for the time course (Figure 4B). To confirm the role of CD36 in the activation of PDE3A, experiments were repeated with oxPCCD36. In human platelets, oxPCCD36 (25 μM), but not PAPC (25 μM), increased PDE3A activity (26.9±7.3%; $P<0.05$ compared to basal and PAPC) (Figure 4C). Critically, oxPCCD36 stimulated activity in WT platelets to 25.4±2.4% ($P<0.05$ compared to basal or PAPC) but not in $CD36^{-/-}$ platelets (Figure 4D).

We previously described a CD36-specific signaling pathway that includes the sequential activation of Src-family kinases (SFK), Syk, PLCγ2 and protein kinase C (PKC)[17] and investigated whether these kinases were involved in the activation of PDE3A. The non-selective SFK inhibitor, dasatinib (10 μM), ablated oxPCCD36-induced PDE3A activation (Figure 4E), while the Syk inhibitor, R406 (1 μM), caused significantly reduced PDE3A activity (Figure 4F). Given that CD36 signaling leads to PKC activation in a SFK manner, and that PDE3A is a target of PKC in platelets,[31] we tested the PKC inhibitors RO318220 (10 μM) and BIM1 (10 μM), and the intracellular Ca^{2+} chelator BAPTA-AM (20 μM) (Figure

4G). These three inhibitors blocked PDE3A activity induced by oxLDL, suggesting a PKC-dependent pathway (P<0.05). Platelet PDE3A activation is associated with phosphorylation of key serine residues.[31] We examined two of the most characterised sites, ser[312] (PKA and PKC sensitive) and ser[428] (PKC sensitive). OxLDL led to a time-dependent phosphorylation of ser[428] which peaked at 2 min and was still evident at 10 min, but had no effect on phosphoPDE3Aser[312]. In comparison, thrombin (0.1 U/mL) induced significantly less phosphorylation at ser[428], which peaked at 1 min before returning to basal at 2 min (Figure 4H) but also induced phosphorylation of ser312. These data confirm that oxLDL and its associated oxidized phospholipids require the sequential ligation and activation of CD36, Src, Syk and PKC to activate PDE3A.

Dyslipidemia is associated with platelet prostacyclin hyposensitivity in mice

To demonstrate the functional importance of our observations on dyslipidemia-driven thrombosis, we examined platelet sensitivity to PGI_2 in mice fed a Western diet (45%). cAMP signaling, integrin αIIbβ3 activation and thrombosis were assessed in whole blood *ex vivo*, which allowed us to evaluate platelet function and thrombotic potential while avoiding any confounding effects of altered PGI_2 synthesis *in vivo* associated with dyslipidemia.[32] Western diet significantly raised cholesterol levels (*Online Supplementary Figure S8*) and the presence of oxidized phospholipids in the plasma (Figure 5A). We then investigated the effect of dyslipidemia on cAMP signaling using multiplexed phosphoflow cytometry to allow the examination of signaling in the physiological conditions of whole blood.[24] Platelets from high-fat fed WT animals produced significantly less phosphoVASP-ser[157] when challenged with PGI_2 (100 nM) than normal chow WT mice (4.9±0.4 fold *vs.* 6.2±0.2-fold increase over basal; P<0.05) (Figure 5B). The deletion of CD36 protected cAMP signaling in dyslipidemic blood, with phosphoVASP-ser[157] remaining at control levels (Figure 5B). In parallel experiments, blood was stimulated with CRP-XL (10 μg/mL) in the presence and absence of PGI_2 (100 nM), and αIIbβ3 activation was measured. In normal chow WT blood, PGI_2 caused 75.7±3.9% inhibition of integrin activation (P<0.05 compared to absence of PGI_2), which was blunted in high-fat fed WT blood (43.1%±7.6 inhibition, P<0.05 compared to normal chow) (Figure 5C, left). Conversely, in CD36$^{-/-}$ blood, PGI_2 induced inhibition of integrin activation was not significantly different in normal chow and high-fat fed conditions (66.5±8% and 79.3±6.5%, respectively) (Figure 5C, 1st and 2nd left).

When we assessed *ex vivo* thrombosis under flow, normal chow WT blood formed small thrombi on immobilized collagen in a time dependent manner, which was abolished by PGI_2 (20 nM) (Figure 5Di). High-fat fed WT blood showed an accelerated thrombotic response with increased surface area (11±3.6% compared to 16.2±4.3% at 2 mins). In addition, dyslipidemia caused significant hyposensitivity to PGI_2, with the prostanoid causing 31.7±10.7% inhibition compared 61.6±5.6% with normal chow (P<0.05; 2 min) (Figure 5ii and iii). In contrast, accelerated platelet deposition on collagen and platelet hyposensitivity to PGI_2 was not detected in CD36$^{-/-}$ high-fat fed blood (Figure 5D). We repeated the experiments with 8-CPT-6-Phe-cAMP. If PDE3A activation was responsible for the increased thrombotic potential associated with reduced sensitivity to PGI_2, then CPT-6-Phe-cAMP-mediated inhibition of thrombosis would be unaffected by dyslipidemia. The cAMP analog caused a similar degree of inhibition of thrombosis in all genotypes but critically remained unaffected in the context of dyslipidemia (WT-normal chow, 65.6±11.2%; WT-Western diet, 62.3±7.7%; CD36$^{-/-}$-normal chow, 70.4±2.0%; CD36$^{-/-}$-Western diet, 62.3±5% inhibition) (*Online Supplementary Figure S9*). Here we show a physiological role for platelet CD36 in dyslipidemia, where it drives a phenotype of platelet hyperactivity by blocking cAMP-mediated inhibition.

Oxidized low density lipoprotein potentiation of thrombosis *in vivo* is prevented by inhibition of phosphodiesterase 3A

To examine the role of oxLDL in thrombosis *in vivo* we used intravital microscopy following ferric chloride–induced carotid artery injury. Tail-vein injections of oxLDL (2.5 mg/kg^{-1} bodyweight)[33,34] into WT mice accelerated post-injury thrombotic response at all time points compared to control PBS injection (Figures 6A-C and *Online Supplementary Videos S1* and *S2*). Next animals were pre-treated with milrinone to explore in principle whether PDE3A inhibition might diminish prothrombotic effects of oxLDL. Consistent with previous studies, modulation of PDE3A activity reduced murine thrombosis (Figure 6A-C).[23] Importantly, the presence of milrinone (10 μmol/L) significantly reduced the ability of oxLDL to promote thrombosis at all time points post injury (P<0.05) (*Online Supplementary Video S3*) suggesting the heightened thrombotic response in the presence of oxLDL could be related, at least in part, to changes in PDE3A activity.

Discussion

The presence of oxidized lipid epitopes, including oxLDL, is thought to promote platelet hyperactivity in subjects with obesity, CAD and stroke.[5,6,35] It has been established that oxidative modifications are a hallmark of dyslipidemia and that they stimulate platelet activation directly through a number of distinct receptors.[6,8,13] Interestingly, platelets from patients with CAD and dyslipidemia show a reduced sensitivity to the inhibitory effects of PGI_2. These observations, coupled to pharmacological trials indicating that suppression of endothelial PGI_2 synthesis increased rate of atherothrombotic events,[36–39] suggest that platelet sensitivity to PGI_2 could play an undefined role in the development of atherothrombotic events. Hence, the aim of this study was to investigate whether oxLDL may promote unwanted platelet activation through the modulation of platelet sensitivity to PGI_2. Using a combination of pharmacological and genetic approaches, we show that oxidative lipid stress modulates platelet cAMP signaling leading to increased platelet activation. Our key findings include: (i) oxLDL and oxidized phospholipids decrease platelet sensitivity to PGI_2, which is coupled to reduced platelet accumulation of cAMP and PKA mediated signaling; (ii) PGI_2 hyposensitivity likely occurs *via* sustained activation of the cAMP hydrolysing enzyme PDE3A in response to oxLDL; (iii) the activation of PDE3A by oxLDL requires ligation of CD36; and (iv) that dyslipidemia induces platelet hyposensitivity to PGI_2 in a CD36-dependent manner.

In the first instance, we used three increasingly physiological systems to show that exposure of platelets to oxLDL opposes the inhibitory effects of PGI_2. In contrast, oxLDL failed to affect platelet inhibition by 8-CPT-6-Phe-cAMP, a PDE-resistant cAMP analog, demonstrating firstly that the PKA signaling was intact and secondly that the effects of the oxidized lipoprotein may regulate cAMP availability. Exploration of the underlying mechanisms demonstrated that OxLDL prevented the accumulation of cAMP in response to both PGI_2 and forskolin. Forskolin increases cAMP in a receptor-independent manner, thereby providing evidence that oxLDL did not affect the interaction of PGI_2 with the IP receptor or target adenylyl cyclases. This is consistent with previous studies demonstrating that reduced platelet sensitivity to PGI_2 in patients with hypercholesterolemia was independent of any changes in cAMP synthesis by adenylyl cyclase.[36] It was therefore possible that oxLDL could either prevent the synthesis of cAMP or accelerate its breakdown. We further found that oxLDL failed to modulate cAMP concentrations in the presence of the PDE3 inhibitor milrinone, but not the PDE2 inhibitor EHNA, suggesting that cAMP hydrolysis by PDE3A was the potential mediator of PGI_2 hyposensitivity. A role for PDE3A was confirmed using immunoprecipitation experiments showing that both oxLDL and oxPCCD36 accelerated the hydrolytic activity of PDE3A in both human and murine platelets through ligation of CD36. The activation of PDE3A downstream of CD36 required the activation of Src family kinases, Syk and PKC. This provides further evidence that CD36-SFK-Syk represents a multiprotein complex that transduces extracellular oxidative lipid stress to the intracellular signaling machinery of the platelet. Interestingly, hemostatic agonists such as thrombin and collagen also activate PDE3A through a PKC-dependent mechanism.[31] These agonists are proposed to cause a rapid attenuation of cAMP signaling at sites of vascular injury to promote platelet-mediated hemostasis. However, in contrast to the rapid and short-lived activation of PDE3A activity by thrombin and collagen, oxLDL induced a sustained PDE3A response for up to 60 min (longest time tested). This was linked to a different activatory phosphorylation pattern of PDE3A by oxLDL and could suggest a distinct mechanism of activation induced by short-lived hemostatic agonists from that of oxLDL. Given the sustained activation of platelet PDE3A in the presence of oxidative lipid stress, it is attractive to speculate that PDE3A may be partially activated in dyslipidemic disease states and thereby reduce the threshold for platelet activation by diminution of cAMP. Indeed, gain of function mutations of PDE3 are associated with stroke, underlining its role in vascular pathology.[40] This concept is also supported by observations that inhibition of PDE3A by cilostazol can have beneficial anti-thrombotic effects in high-risk groups characterized a prothrombotic phenotype.[41–43]

The pathophysiological consequences of platelet hyposensitivity to PGI_2 and the potential importance of CD36 was explored in a murine model of high-fat feeding-induced dyslipidemia. Interestingly, we found that even mild dyslipidemia was characterized by the presence of oxidized lipid epitopes in the plasma, which was unaffected by the absence of CD36. Whole blood phosphoflow cytometry was used to measure platelet phosphoVASP, as a marker of cAMP signaling, without the need for cell isolation. This demonstrated that mild dyslipidemia was accompanied by reduced cAMP signaling. The functional importance of this blunted cAMP signaling response manifested as diminished platelet sensitivity to the inhibitory effects of PGI_2 on integrin activation measured by flow cytometry and *ex vivo* thrombosis. The assessment of thrombosis *ex vivo* was important to demonstrate that hyposensitivity of platelets to PGI_2 was a primary platelet defect rather than a response to a dysfunctional endothelium, where altered PGI_2 production has been observed in models of dyslipidemia.[32] To support our hypothesis that hyposensitivity to PGI_2 was linked to PDE3A activity, we showed that cAMP signaling in dyslipidemia was normal if a PDE-resistant cAMP analog (8-CPT-6-Phe-cAMP) was used, again confirming that PKA signaling downstream of cAMP was functional. Critically, genetic ablation of CD36 protected animals from the loss of PGI_2 sensitivity and restored PKA signaling. Infusions of oxLDL into wild-type mice caused a robust potentiation of thrombosis by ferric chloride. However, mice were protected from the prothrombotic effects of oxLDL *in vivo* when PDE3A was pharmacologically inhibited. Using this approach, milrinone did not target only platelets and could therefore have an effect on other PDE3A expressing cells. However, the data are proof of principle that the prothrombotic effects of oxLDL *in vivo*, at least in part, may be prevented by therapeutic strategies based on enhancing or preserving cAMP signaling events in platelets. This element of the work requires further studies focussing on strategies for the specific targeting of PDE3A, and potentially PDE2, in platelets.

Together, our *ex vivo* and *in vitro* data suggest a previously unrecognized mechanism contributing to platelet hyperactivity, where the ligation of CD36 by oxidized lipids modulates cAMP signaling by activating PDE3A leading to PGI_2 hyposensitivity. These data may constitute a link to the observed PGI_2 hyposensitivity in dyslipidemic high-risk populations and indicate a novel therapeutic strategy to target atherothrombotic risk in certain patient groups. Remarkably, current antiplatelet therapy exclusively targets platelet activatory pathways including cyclo-oxygenases (Aspirin), P2Y12 (Thienopyridines, non-Thienopyridines) or $\alpha_{IIb}\beta_3$ (Tirofiban; Eptifibatide) while platelet inhibitory pathways remain untargeted. Therefore, high-risk populations might remain at increased atherothrombotic risk despite optimal available pharmacological therapy, and impaired platelet inhibition might contribute to the residual cardiovascular risk.

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References

1. Mitchell JA, Ali F, Bailey L, Moreno L, Harrington LS. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. Exp Physiol. 2008;93(1):141-147.
2. Jackson SP. Arterial thrombosis--insidious, unpredictable and deadly. Nat Med. 2011; 17(11):1423-1436.
3. Davì G, Romano M, Mezzetti A, et al. Increased levels of soluble P-selectin in hypercholesterolemic patients. Circulation. 1998;97(10):953-957.
4. Carvalho AC, Colman RW, Lees RS. Platelet function in hyperlipoproteinemia. N Engl J Med. 1974;290(8):434-438.
5. Colas R, Sassolas A, Guichardant M, et al. LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. Diabetogia. 2011;54(11):2931-2940.
6. Chan H-CC, Ke L-YY, Chu C-SS, et al. Highly electronegative LDL from patients with {ST-elevation} myocardial infarction triggers platelet activation and aggregation. Blood. 2013;122(22):3632-3641.
7. Ardlie NG, Selley ML, Simons LA. Platelet activation by oxidatively modified low density lipoproteins. Atherosclerosis.1989;76(2–3):117-124.
8. Chen R, Chen X, Salomon RG, M, McIntyre T. Platelet activation by low concentrations of intact oxidized LDL particles involves the PAF receptor. Arterioscler. Thromb Vasc Biol. 2009;29(3):363-371.
9. Naseem KM, Goodall AH, Bruckdorfer KR. Differential effects of native and oxidatively modified low-density lipoproteins on platelet function. Platelets. 1997;8(2–3):163–173.
10. van Willigen G, Gorter G, Akkerman JW. LDLs increase the exposure of fibrinogen binding sites on platelets and secretion of dense granules. Arterioscler Thomb. 1994;14(1):41–46.
11. Podrez EA, Byzova T V, Febbraio M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nat Med. 2007;13(9):1086–1095.
12. Korporaal S, Eck M, Adelmeijer J, et al. Platelet Activation by Oxidized Low Density Lipoprotein Is Mediated by Cd36 and Scavenger {Receptor-A}. Arterioscler. Thromb Vasc Biol. 2007;27(11):2476–2483.
13. Chen K, Febbraio M, Li W, Silverstein RL. A Specific CD36-Dependent Signaling Pathway Is Required for Platelet Activation by Oxidized Low-Density Lipoprotein. Circ Res. 2008;102(12):1512–1519.
14. Wraith KS, Magwenzi S, Aburima A, et al. Oxidized low-density lipoproteins induce rapid platelet activation and shape change through tyrosine kinase and Rho kinase-signaling pathways. Blood. 2013; 122(4):580–589.
15. Biswas S, Zimman A, Gao D, Byzova T V, Podrez EA. TLR2 Plays a Key Role in Platelet Hyperreactivity and Accelerated Thrombosis Associated With Hyperlipidemia. Circ Res. 2017;121(8):951–962.
16. Chen K, Li W, Major J, et al. Vav guanine nucleotide exchange factors link hyperlipidemia and a prothrombotic state. Blood. 2011;117(21):5744–5750.
17. Magwenzi S, Woodward C, Wraith KS, et al. Oxidized LDL activates blood platelets through CD36/NOX2-mediated inhibition of the cGMP/protein kinase G signaling cascade. Blood. 2015;125(17):2693–2703.
18. Yang M, Cooley BC, Li W, et al. Platelet CD36 promotes thrombosis by activating redox sensor ERK5 in hyperlipidemic conditions. Blood. 2017;129(21):2917–2927.
19. Yang M, Kholmukhamedov A, Schulte ML, et al. Platelet CD36 signaling through ERK5 promotes caspase-dependent procoagulant activity and fibrin deposition in vivo. Blood Adv. 2018;2(21):2848–2861.
20. Raslan Z, Naseem KM. The control of blood platelets by cAMP signalling. Biochem. J Trans. 2014;42(2):289–294.
21. Beck F, Geiger J, Gambaryan S, et al. Time-resolved characterization of cAMP/PKA-dependent signaling reveals that platelet inhibition is a concerted process involving multiple signaling pathways. 2014;123(5):e1–e10.
22. Beca S, Ahmad F, Shen W, et al. Phosphodiesterase type 3A regulates basal myocardial contractility through interacting with sarcoplasmic reticulum calcium {ATPase} type 2a signaling complexes in mouse heart. Circ Res. 2013;112(2):289–297.
23. Sim DS, Glenn M-S, Furie BC, Furie B, Flaumenhaft R. Initial accumulation of platelets during arterial thrombus formation in vivo is inhibited by elevation of basal cAMP levels. Blood. 2004;103(6):2127–2134.
24. Spurgeon BE, Aburima A, Oberprieler NG, Taskén K, Naseem KM. Multiplexed phosphospecific flow cytometry enables large-scale signaling profiling and drug screening in blood platelets. J Thromb Haemost. 2014;12(10):1733–1743.
25. Haslam RJ, Dickinson NT, Jang EK. Cyclic nucleotides and phosphodiesterases in platelets. Thromb Haemost. 1999; 82(2):412–423.
26. Conti M, Beavo J. Biochemistry and Physiology of Cyclic Nucleotide Phosphodiesterases: Essential Components in Cyclic Nucleotide Signaling. Annu Rev Biochem. 2007;76(1):481–511.
27. Butt E, Abel K, Krieger M, et al. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem. 1994;269(20):14509–14517.
28. Chen K, Febbraio M, Li W, Silverstein RL. A specific CD36-dependent signaling pathway is required for platelet activation by oxidized low-density lipoprotein. Circ Res. 2008;102(12):1512–1519.
29. Podrez E a, Byzova T V, Febbraio M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nat Med. 2007;13(9):1086–1095.
30. Butt E, Abel K, Krieger M, Palm D, et al. cAMP-and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem. 1994;269(20):14509-14517.
31. Hunter RW, Carol M, Hers I. Protein Kinase C-mediated Phosphorylation and Activation of PDE3A Regulate {cAMP} Levels in Human Platelets. J Biol Chem. 2009;284(18):12339–12348.
32. Csányi G, Gajda M, Magdalena F-Z, et al. Functional alterations in endothelial NO, PGI_2 and EDHF pathways in aorta in {ApoE/LDLR-/- mice. Prostaglandins Other Lipid Mediat. 2012;98(3–4):107–115.
33. Nakano A, Kawashima H, Miyake Y, et al. 123I–Labeled oxLDL Is Widely Distributed Throughout the Whole Body in Mice. Nucl Med Mol Imaging. 2018;52(2):144-153.
34. Badrnya S, Schrottmaier WC, Kral JB, et al. Platelets mediate oxidized low-density lipoprotein-induced monocyte extravasation and foam cell formation. Arterioscler. Thromb Vasc Biol. 2014;34(3):571–580.
35. Shen M-YY, Chen F-YY, Hsu J-FF, et al. Plasma L5 levels are elevated in ischemic stroke patients and enhance platelet aggregation. Blood. 2016;127(10):1336–1345.
36. Colli S, Lombroso M, Maderna P, Tremoli E, Nicosia S. Effects of PGI_2 on platelet aggregation and adenylate cyclase activity in human type {IIa} hypercholesterolemia. Biochem Pharmacol. 1983;32(13):1989–1993.
37. Sinzinger H, Schernthaner G, Kaliman J. Sensitivity of platelets to prostaglandins in cornary heart disease and angina pectoris. Prostaglandins. 1981;22(5):773–781.
38. Mehta J, Mehta P, Conti CR. Platelet function studies in coronary heart disease. {IX.} Increased platelet prostaglandin generation and abnormal platelet sensitivity to prostacyclin and endoperoxide analog in angina pectoris. Am J Cardiol. 1980;46(6):943–947.
39. Furberg CD, Psaty BM, A FG. Parecoxib, valdecoxib, and cardiovascular risk. Circulation. 2005;111(3):249.
40. Malik R, Chauhan G, Traylor M, et al. Multiancestry genome-wide association study of 520,000 subjects identifies 32 loci associated with stroke and stroke subtypes. Nat Genet. 2018;50(4):524–537.
41. Araki S, Matsuno H, Haneda M, et al. Cilostazol attenuates spontaneous microaggregation of platelets in type 2 diabetic patients with insufficient platelet response to aspirin. Diabetes Care. 2013;36(7):e92-3.
42. Park KW, Kang S-H, Park JJ, et al. Adjunctive Cilostazol Versus Double-Dose Clopidogrel After Drug-Eluting Stent Implantation. JACC Cardiovasc Interv. 2013;6(9):932–942.
43. Angiolillo DJ, Capranzano P, Ferreiro JL, et al. Impact of adjunctive cilostazol therapy on platelet function profiles in patients with and without diabetes mellitus on aspirin and clopidogrel therapy. Thromb Haemost. 2011;106(2):253–262.