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

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SUPPLEMENTARY METHODS.

Platelet preparation

Human washed platelets (WP) were isolated from blood taken from drug-free volunteers as previously described. [1] All Human work was approved by the Hull York Medical School Ethics Committee. Human blood was taken from drug-free volunteers by clean venepuncture using acid citrate dextrose (ACD; 29.9mM sodium citrate, 113.8mM glucose, 72.6mM sodium chloride and 2.9mM citric acid, pH 6.4) as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200g at 20°C for 20minutes. PRP was treated with citric acid (0.3M) and centrifuged at 800g for 12 minutes. The platelet pellet was then suspended in wash buffer (36mM citric acid, 10mM EDTA, 5mM glucose, 5mM KCl, 9mM NaCl) and spun once more at 800g for 12 minutes. Platelets were finally re-suspended at the indicated concentration in modified Tyrodes buffer (150mM NaCl, 5mM HEPES, 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$, 5.6mM glucose, pH7.4).
Murine blood obtained by cardiac puncture was taken into PPACK by under terminal CO_2 narcosis. PRP was obtained by centrifugation of whole blood at 300*g* for 10min at room temperature. The PRP was then centrifuged at 1000*g* after addition of citric acid (0.3M) for 6minutes at room temperature. The pellet was resuspended in modified Tyrodes buffer, spun again at 1000g for 6minutes and then finally resuspended at the indicated concentration in modified Tyrodes buffer. When required for signalling experiments, platelets were incubated with ethylene-glycol-bis-tetraacetic acid (EGTA; 1mM), indomethacin (10μM) and apyrase (2U/ml) for 15 minutes before experimentation.

Platelet aggregation

WP (2.5×10^8 platelets/mL) were incubated with oxLDL/LDL (50μg/ml) at 37°C for 2 minutes followed PGI_2 (20nM) for 1 minute and then the addition of thrombin (0.05U/mL) or collagen (5μg/ml) and aggregation monitored under constant stirring for 4 minutes using a Chronolog Dual Channel Platelet Aggregometer.

Platelet flow assays

For the i*n vitro* thrombosis assay human whole blood was incubated with $DiOC_6$ (1μM) and treated with oxLDL/LDL (50μg/mL) in the presence and absence of PGI_2 (20nM). Assays were performed using Vena8 biochips (Cellix; Dublin, Ireland), coated with collagen (50μg/ml) for 12 hours, and blocked with BSA (10mg/mL) for 1hour. For *ex vivo* experiments murine blood was treated in the same manner except the addition of nLDL/oxLDL was omitted. Flow was performed for 2minutes at $1000s^{-1}$. Images of stably adhered platelets and thrombi were captured using fluorescence microscopy and analysed using ImageJ software. Data are presented as surface area coverage (%), since the software could not fully discriminate between single platelets and platelet aggregates. [2]

Flow cytometric analysis

Flow cytometry assays were performed with whole blood from either mice or human subjects. For human studies, whole blood was incubated with oxLDL/LDL (50μg/ml) at 37°C for 2 minutes, followed by PGI_2 (20nM) for 1 minute and the assay initiated by the addition of PAR-1 peptide (10μM). Flow cytometry was performed using a BD LSRFortessa and analysed for P-selectin expression. For murine studies blood was harvested and examined for phosphoVASP or JonA binding. For PhosphoVASP a phosphoflow cytometry approach was used. In brief, whole blood was incubated with PGI_2 (20nM) for 1 minute. Cells were then pelleted by centrifugation at 1000*g at* 4°C, for 10min, the supernatants removed and the remaining platelets permeabilised for 10 min with 0.1% Triton X-100 in phosphate buffered saline (PBS). Permeabilised platelets were pelleted (1000*g at* 4°C, for 10minutes) washed with PBS and incubated with phosphospecific antibodies (2μg/mL) on ice for 30 minutes. Platelets were washed with PBS and incubated with a secondary antibody labeled with AlexaFluor 647 (1μg/mL) on ice in the dark for 45 minutes. After washing with PBS, platelets were transferred to FACS tubes for flow cytometric analyses using a BD LSRFortessa. [3] For JON/A binding platelet were incubated with PGI_2 (100nM) for 1 minute followed by addition of CRP-XL (10μg/ml) for 5minutes. After fixation platelets were stained and processed using a BD LSRFortessa

Immunoblotting

Washed platelets (5×10^8 platelets/mL), incubated with apyrase (2U/mL), indomethacin (10μmol/L) and EGTA (1mmol/L), were treated with oxLDL/nLDL (50μg/ml for 15min) prior to the addition of PGI_2 (20nM) for 1 minute at 37°C before termination with Laemmli buffer. In some cases, platelets were incubated with the CD36 blocking antibody FA6-152 or IgG (1μg/mL), for 20min at 37°C prior to addition of oxLDL. Whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 60minutes with 10% BSA or 5% milk dissolved in Tris-buffered-saline-Tween (0.1%)

(TBS-T) then incubated with anti-phosphoPKA substrate (1:1000), anti-phosphoVASP-ser^{239} (1:1000), anti-phosphoVASP-ser^{157} (1:1000), anti-pDE3Aser^{428} (1:250), anti-pDE3Aser^{312} (1:250) or an anti β-tubulin antibody (1:1000). Membranes were developed with enhanced chemiluminescence (ECL) solutions.

cAMP measurement

Intracellular cAMP concentrations were quantified using an enzyme immunoassay (EIA) (GE Healthcare). cAMP is measured through the competition between cAMP in a test sample and a fixed quantity of peroxidase-labeled cAMP, for a limited number of binding sites on a cAMP specific antibody. WPs ($2x10^8$/ml) were treated with the indicated agents for selected time intervals and lysed with 10x lysis buffer 1A (2.5% dodecyltrimethylammonium in assay buffer). An aliquot (100µl) of each sample was added to a 96 well plate in triplicate. After a 2 hour incubation at 4°C with gentle shaking, cAMP-peroxidase conjugate (50µl) was then added to each well and incubated for 1 hour at 4°C with gentle shaking. Following incubation, the wells were then washed four times and hydrogen peroxide substrate was added to each well. Absorbance was read at 630nm using a TECAN infinite M200 microplate reader.

Measurement of PDE3A activity

To measure intracellular PDE activity we used a commercially available non-radioactive colorimetric assay. WPs ($5x10^8$/ml) were treated as described, lysed in 2x PDE extraction buffer (150mM NaCl, 50mM HEPES, 20% glycerol (v/v), 10% Igepal (v/v), 1mM, EDTA, 1:200 phosphatase inhibitor cocktail (v/v), protease inhibitor cocktail 1:100 (v/v)) and immediately placed on ice. PDE3A was immunoprecipitated from 500µg of protein lysate using 1µg of anti-PDE3A antibody or matched IgG control. Immunoprecipitates were incubated with 5'-nucleotidase and of cAMP (0.5mM) substrate at 37°C for 1 hour and the production of 5'-AMP measured following the manufacturers instructions. Activity was expressed as fmol AMP/min $1x10^7$ platelets and normalised to control values to account for variations in basal activity between individual platelet donors.

Intravital imaging of in vivo thrombosis

Left carotid artery of anaesthetised mice was exposed and mice injected with Rhodamine G followed by either oxLDL (2.5mg/kg^{-1} body weight) or an equal volume of PBS through the tail vein. [4,5] In some mice, milrinone (10µmol/L) was injected 5 minutes prior to oxLDL injection. 10 minutes after the injection of oxLDL, injury was induced by applying 1x2mm filter paper saturated with anhydrous $FeCl_3$ (10%). Filter paper was placed on the right adventitial surface of the vessel for 1minute then removed. Thrombosis was recorded using high-speed intravital microscopy for up to 40 minutes. The integrated intensity value (Median Fluorescence Intensity) of the thrombus was measured over time and time taken to reach peak was determined. Five individual mice were used for each condition.

Statistics.

Results are expressed as means ± SEM and statistical analyses were undertaken using Prism 6.0 (GraphPad, La Jolla, California). Comparisons between groups were performed by an unpaired, Mann-Whitney U test. P values of less than 0.05 were considered statistically significant.

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SUPPLEMENTARY FIGURES
Supplementary Material

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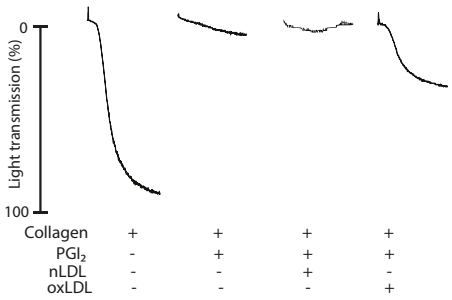

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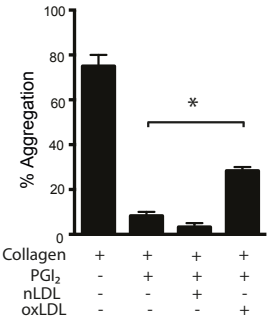

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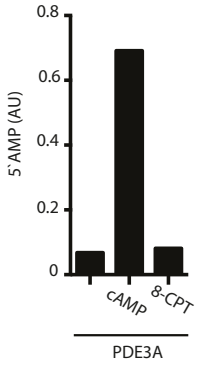

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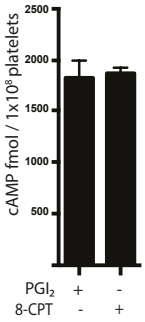

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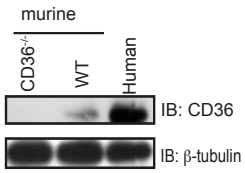

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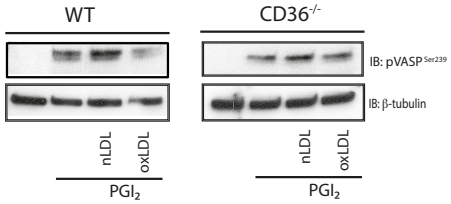

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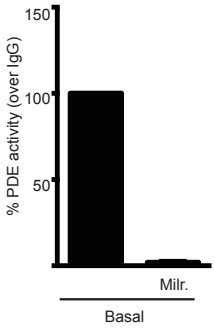

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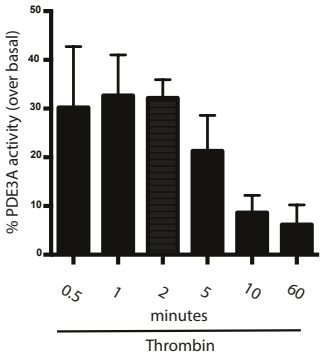

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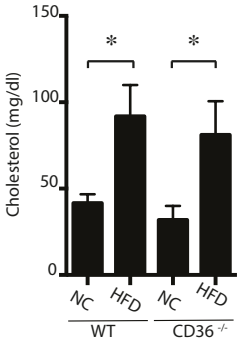

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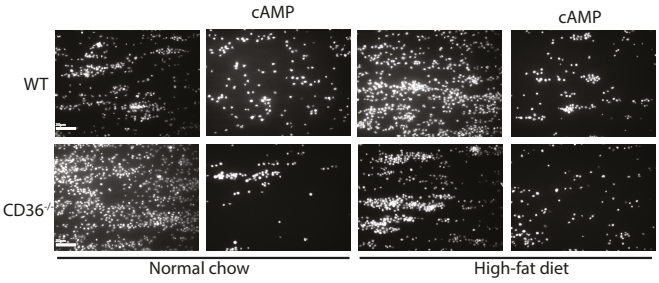

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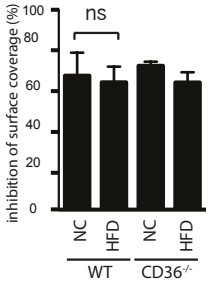

Supplementary Figure 1: OxLDL induces PGI_2 hyposensitivity in platelets.
Washed human platelets (2.5 x 10^8/ml) were incubated with nLDL or oxLDL (50μg/ml) for 2 minutes followed by PGI_2 (20nM) for 1 minute. Collagen-stimulated aggregation was then measured under constant stirring (1000 rpm) at 37°C for 4 minutes. (i) Representative aggregation traces (ii) Data is expressed as percentage aggregation and presented as mean ± SEM (n= 5, * $p < 0.05$ compared to PGI_2 alone).

Supplementary Figure 2: 8-CPT-6-Phe-cAMP is resistant to PDE3a.
PDE3A immunoprecipitates were incubated with cAMP and 8-CPT-6-Phe-cAMP and enzyme activity was measured by production of 5'AMP after 1hour at 37°C. N=2

Supplementary Figure 3: 8-CPT-6-Phe-cAMP increases intracellular cAMP.
Platelets were incubated with PGI_2 (50nM) or 8-CPT-6-Phe-cAMP (50μM) for 5 minutes. Platelets were washed, lysed and intracellular cAMP levels were determined by enzyme immunoassay. Data is presented as mean ± SEM.

Supplementary Figure 4: Immunoblotting of platelet lysates for CD36
Washed human platelets, WT murine platelets and $CD36^{-/-}$ platelets ($5x10^8$/ml) were lysed with Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-CD36. Representative blot of 3 independent experiments

Supplementary Figure 5: OxLDL modulation of $VASP^{239}$ phosphorylation in murine platelets
Washed murine WT or $CD36^{-/-}$ platelets ($5x10^8$/ml) were treated alone or with nLDL or oxLDL (50μg/ml) for 2 minutes followed by a 1 minute PGI_2 (50nM) incubation. Treated platelets were lysed with Laemmli buffer, separated by SDS-PAGE and immunoblotted with anti-phosphoVASPser^{239} or anti-β tubulin. Representative blot of 3 independent experiments.

Supplementary Figure 6: Immuniprecipitated phosphodiesterase is sensitive to milrinone.
Washed human platelets ($2.5x10^8$/ml) lysed and PDE3A was immunoprecipitated. Enzyme activity measured by cAMP hydrolysis for 1hour at 37°C in the presence and absence of Milrinone (20μM). Data is presented as % activity with basal representing 100% (n=3).

Supplementary Figure 7: Thrombin induced activation of PDE3A as a function of time.
Washed human platelets ($2.5x10^8$/ml) stimulated with thrombin (0.1U/ml) for up to 60 minutes before lysis. PDE3A was immunoprecipitated and enzyme activity measured by cAMP hydrolysis for 1hour at 37°C. Data is presented as % over basal activity (n=3).

Supplementary figure 8: Plasma cholesterol concentrations from WT and $CD36^{-/-}$ mice.
WT and $CD36^{-/-}$ mice were fed either normal chow or High fat diet. Plasma was isolated from whole blood and the concentration of cholesterol measured. Data is expressed as mg/dl cholesterol and presented as mean ± SEM (n=5). * $p<0.05$).

Supplementary figure 9: High-fat diet fed animals show normal platelet sensitivity to PDE-resistant cAMP analogue.
Whole blood of normal chow and high-fat diet fed animals treated with 8-CPT-6-Phe-cAMP (50μM) for 5 minutes was perfused at arterial shear $1000s^{-1}$ for 2 minutes over a collagen (50μg/ml). Images of adherent platelets were taken by fluorescence microscopy. (i) Representative images of arterial flow experiments, (ii) Data expressed as percentage inhibition of surface coverage, mean ± SEM (n=5; ns = not significant).