

## ORIGINAL ARTICLE



# Dyslipidemia-associated atherogenic oxidized lipids induce platelet hyperactivity through phospholipase C $\gamma$ 2-dependent reactive oxygen species generation

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## Abstract

Oxidized low-density lipoprotein (oxLDL) and associated oxidized phosphocholine-headgroup phospholipids (oxPCs) activate blood platelets through ligation of the scavenger receptor CD36. Previously, we found that oxLDL stimulated phosphorylation of phospholipase C $\gamma$ 2 (PLC $\gamma$ 2). However, the functional relevance of PLC $\gamma$ 2 phosphorylation in oxLDL-mediated platelet hyperactivity remained elusive. Here, we set out to explore the functional importance of PLC $\gamma$ 2 in oxLDL-mediated platelet activation using human and genetically modified murine platelets. The CD36-specific oxidized phospholipid (oxPC<sub>CD36</sub>) triggered the generation of reactive oxygen species (ROS) in platelets under static and arterial flow conditions. The ROS generation in response to oxPC<sub>CD36</sub> was sustained for up to 3 h but ablated in CD36- and PLC $\gamma$ 2-deficient platelets. The functional importance of ROS generation in response to atherogenic lipid stress was examined through measurement of P-selectin expression. OxPC<sub>CD36</sub> induced P-selectin expression, but required up to 60 min incubation, consistent with the timeline for ROS generation. P-selectin expression was not observed in CD36- and PLC $\gamma$ 2-deficient mice. The ability of oxPC<sub>CD36</sub> and oxLDL to stimulate P-selectin expression was prevented by incubation of platelets with the ROS scavenger N-acetyl-cysteine (NAC) and the NOX-2 inhibitor gp91ds-tat, but not with the NOX-1 inhibitor ML171. In summary, we provide evidence that prolonged exposure to oxLDL-associated oxidized phospholipids induces platelet activation via NOX-2-mediated ROS production in a CD36- and PLC $\gamma$ 2-dependent manner.

## Keywords

OxLDL, oxPC<sub>CD36</sub>, platelet hyperactivity, PLC $\gamma$ 2, reactive oxygen species

## History

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## Introduction

Studies in mice and humans suggest strongly that dyslipidemia induces a prothrombotic phenotype in which platelet hyperactivity plays a major role (1–3). Dyslipidemia is characterized by increased levels of atherogenic oxidized lipid-rich particles, which are proposed as potential mediators of altered platelet function (2–4). Oxidized low-density lipoprotein (oxLDL) and oxidized phosphocholine-headgroup phospholipids (oxPCs) represent sterile danger-associated molecular patterns that promote platelet hyperactivity in dyslipidemia and coronary artery disease (2,3,5). Our work, and that of others, has shown that the scavenger receptor CD36 acts to functionally link oxLDL and oxPCs to platelet activation (2,6–9). However, the detailed mechanisms driving platelet activation in response to the oxLDL–CD36 signaling node are yet to emerge. We recently demonstrated that NOX-2-dependent reactive oxygen species (ROS) production by oxLDL and oxPCs leads to CD36-mediated

platelet hyperactivity by reducing sensitivity to cGMP signaling (6). Importantly, intracellular ROS serve as essential cell-signaling molecules that drive platelet aggregation, P-selectin expression, platelet–neutrophil interactions, and TxB<sub>2</sub> production (10–13). Previously, we found that the oxLDL–CD36 signaling node is linked to the phosphorylation and activation of PLC $\gamma$ 2 and, therefore, hypothesized that PLC $\gamma$ 2 activation is required for oxLDL-mediated ROS induction (6,7,13). The aim of this study was to determine the role of PLC $\gamma$ 2 in oxLDL- and oxPC<sub>CD36</sub>-induced ROS production and to investigate its functional relevance.

## Methods

### Materials

Native LDL (nLDL) was isolated from drug-free, healthy donors and oxidized as previously described (14). P-Selectin was from BD Bioscience (Oxford, UK), and oxPC<sub>CD36</sub> (KoDiA-PC), 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC), and anti-pPLC $\gamma$ 2 1217 antibody were from Santa Cruz (Wembley, UK). E06 anti-oxPC antibody was from Avanti Polar Lipids (Alabama, USA). Anti- $\beta$ -tubulin was from Merck Millipore (Watford, UK). ROS detection kit and N-acetyl-cysteine (NAC)

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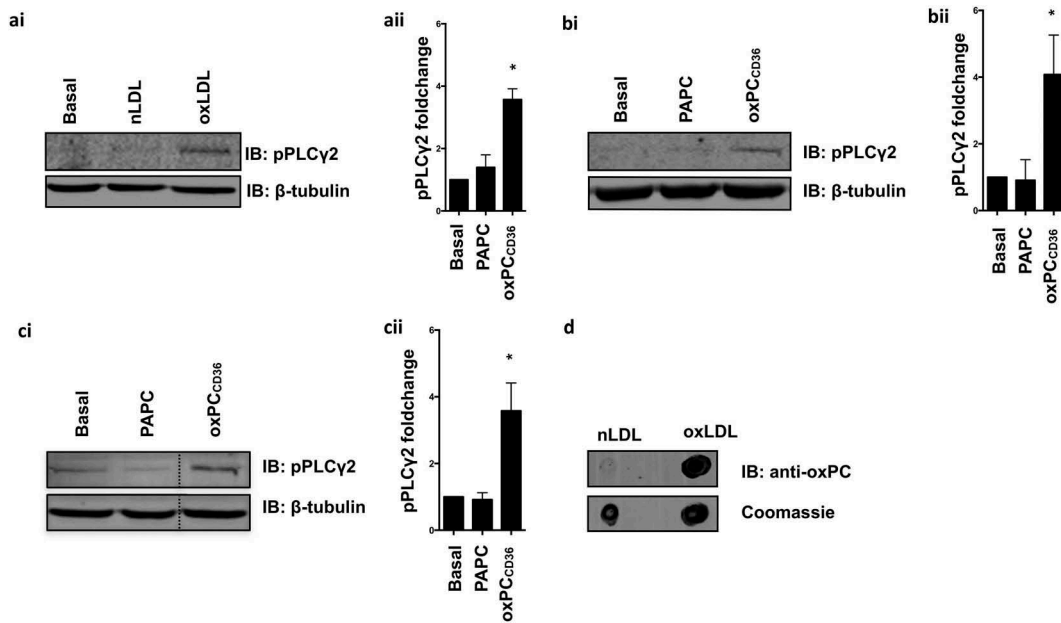


Figure 1. Oxidized LDL and oxidized phospholipids induce phosphorylation of PLC $\gamma$ 2 in human and murine platelets. (ai) Washed human platelets ( $5 \times 10^8$  platelets/ml) incubated with apyrase, indomethacin, EGTA, and tirofiban were treated with nLDL (50  $\mu$ g/ml) or oxLDL (50  $\mu$ g/ml) for 2 min. Lysates were separated by SDS-PAGE and immunoblotted for pPLC $\gamma$ 2-tyr1217 (1:1000) and  $\beta$ -tubulin (1:1000). (aii) Quantification of three independent experiments,  $*p < 0.05$ . (bi) Human platelets treated with oxPC $_{CD36}$  (50  $\mu$ M) or PAPC (50  $\mu$ M) for 15 min analyzed as previously described. (bii) Quantification of three independent experiments,  $*p < 0.05$ . (ci) Murine platelets treated with oxPC $_{CD36}$  (25  $\mu$ M) or PAPC (25  $\mu$ M) for 15 min and analyzed as previously described. (cii) Quantification of three independent experiments,  $*p < 0.05$ . All immunoblots are representative of three independent experiments. (d) nLDL (3  $\mu$ g) and oxLDL (3  $\mu$ g) blotted for oxidized phospholipids by anti-oxPC antibody E06 (1:1000).

was from Enzo Life Sciences (Exeter, UK). ML171 was from Tocris (Bristol, UK). GP91ds-tat was from AnaSpec (Fremont, USA). Collagen was from Takeda (Wien, Austria). Thrombin and indomethacin were from Sigma (Munich, Germany).

### Immunoblotting

All human and animal work was approved by the Hull York Medical School Ethics Committee. Human blood was taken from drug-free volunteers. Human and murine platelets ( $5 \times 10^8$  platelets/ml) from wild-type (WT) and PLC $\gamma$ 2-deficient mice were isolated and lysed as described previously (13). Lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% milk and then incubated with anti-beta tubulin (1:1000) or anti-PLC $\gamma$ 2 pTyr 1217 (1:1000) (6). Membranes were developed and analyzed using a LICOR CLx Imaging System (Cambridge, UK). For dot blots, 3  $\mu$ g of nLDL and oxLDL were loaded onto a PVDF membrane and blotted for anti-oxPC by E06 (1:1000).

### Experimental animals

PLC $\gamma$ 2 $^{-/-}$  and CD36 $^{-/-}$  mice were provided by Professor S. Watson (University of Birmingham, UK) and Professor M. Febbraio, (University of Alberta, Canada), respectively, with WT (C57Bl/6) animals from Charles River (Kent, UK). All mice were used at 8 weeks of age.

### Flow cytometry

Isolation of human and murine platelets was performed as described previously (6). For flow cytometry experiments, platelet-rich plasma (PRP) was incubated for 1 h in the presence or absence of oxLDL (50  $\mu$ g/ml), nLDL (50  $\mu$ g/ml), oxPC $_{CD36}$  (25  $\mu$ M), or PAPC (25  $\mu$ M). In some cases, PRP

was preincubated with NAC (5 mM), gp91ds-tat (2  $\mu$ M), and ML171 (5  $\mu$ M) for 30 min and P-selectin expression was analyzed using a BD LSRFortessa Cytometer.

### Analysis of ROS generation

Washed murine platelets ( $2 \times 10^8$  platelets/ml) were incubated with a previously validated ROS Detection Probe (5  $\mu$ M) at 37°C for 30 min (Enzo Life Sciences, Exeter, UK) and pretreated with oxPC $_{CD36}$  or PAPC (5–25  $\mu$ M) for 15 min before reconstitution with autologous red blood cells (50/50; v/v) (6). Blood was perfused through fibrinogen (1 mg/ml)-coated biochips (Cellix Ltd.) at arterial shear 1000  $s^{-1}$  for 2 min and analyzed for surface coverage and mean fluorescence intensity using ImageJ v2.0. Alternatively, washed murine platelets ( $1 \times 10^8$  platelets/ml) were incubated with an ROS Detection Probe and oxPC $_{CD36}$  for up to 3 h in suspension and fluorescence was measured using a microplate reader (Tecan Group Ltd.).

### Statistics

Data are presented as means  $\pm$  standard error of the mean of at least three independent experiments. Differences between groups were assessed using the Student's *t*-test, Mann-Whitney *U*-test, or ANOVA and statistical significance taken at  $p \leq 0.05$ .

### Results

#### Oxidized LDL and associated oxidized phospholipids induce PLC $\gamma$ 2 phosphorylation

Incubation of human platelets with oxLDL (50  $\mu$ g/ml), but not nLDL (50  $\mu$ g/ml), led to a significant phosphorylation of PLC $\gamma$ 2 (Figure 1(ai) and (aii)). To confirm these findings in murine platelets, we used oxPC $_{CD36}$ , an oxidized phospholipid present in oxLDL (15), to account for potential differences in

sensitivity between human and murine platelets to human oxLDL. Concordantly, oxPC<sub>CD36</sub> (25-50 $\mu$ M), but not the non-oxidized lipid PAPC (25-50 $\mu$ M) mimicked oxLDL induced PLC $\gamma$ 2 phosphorylation on human and murine platelets. The presence of oxPC within our oxLDL preparation was confirmed using the oxPC-specific antibody (E06) (Figure 1(d)) (16).

### oxPC<sub>CD36</sub> induces sustained ROS production in a CD36- and PLC $\gamma$ 2-specific manner

We next explored the functional importance of this phosphorylation event using platelets from genetically modified mice lacking PLC $\gamma$ 2 (Figure 2(a)). Focusing on CD36-dependent signaling, we evaluated the role of PLC $\gamma$ 2 in ROS production under flow and static conditions, which we have shown to be important for platelet hyperactivity (6,7). The perfusion of whole blood from WT or PLC $\gamma$ 2-deficient mice over immobilized fibrinogen led to similar levels of platelet adhesion, which was unaltered by 15 min incubation with oxPC<sub>CD36</sub> (Figure 2(b)). However, treatment of blood with oxPC<sub>CD36</sub>, but not PAPC, led to early accumulation of ROS in WT animals. In contrast, ROS generation in response to oxPC<sub>CD36</sub> was blunted significantly in PLC $\gamma$ 2-deficient mice (Figure 2(c)), suggesting that oxPC<sub>CD36</sub> induces ROS generation in a PLC $\gamma$ 2-dependent manner. We next quantified ROS production in suspended platelets. Unlike the rapid and extensive ROS generation by collagen and thrombin (Supplementary Figure S1),

OxPC<sub>CD36</sub> (25  $\mu$ M) induced slow and sustained ROS production for up to 3 h in WT platelets (longest time tested;  $p < 0.001$ ) (Figure 2(di) and (dii)). In contrast with OxPC<sub>CD36</sub>, the unoxidized phospholipid PAPC did not induce ROS production in WT platelets (Supplementary Figure S2). Treatment of platelets deficient in either CD36 or PLC $\gamma$ 2 with oxPC<sub>CD36</sub> did not lead to ROS production above basal levels. To confirm that genetic deletion of CD36 and PLC $\gamma$ 2 did not interfere with the ability or capacity of platelets to produce ROS, platelets were treated with pyocyanin (250  $\mu$ M) that stimulates ROS production via an acute cyclic non-enzymatic reduction by NAD(P)H (17). ROS generation in response to pyocyanin was not affected by the absence of CD36 or PLC $\gamma$ 2 (Figure 2(e)), suggesting that oxPC<sub>CD36</sub>-induced ROS production specifically requires both CD36 and downstream activation of PLC $\gamma$ 2.

### oxLDL and associated oxPC<sub>CD36</sub> induce P-selectin expression by intracellular production of ROS

Having established that PLC $\gamma$ 2 was essential for intracellular ROS production in response to oxidized lipid stress, we next examined how this affected platelet activation. Incubation of WT murine platelets with oxPC<sub>CD36</sub> (25  $\mu$ M), in the absence of other agonists, caused a significant increase in P-selectin expression ( $p = 0.015$ ), while PAPC (25  $\mu$ M) had no effect (Figure 3 (a)). Again, the ability of the oxidized phospholipid to stimulate

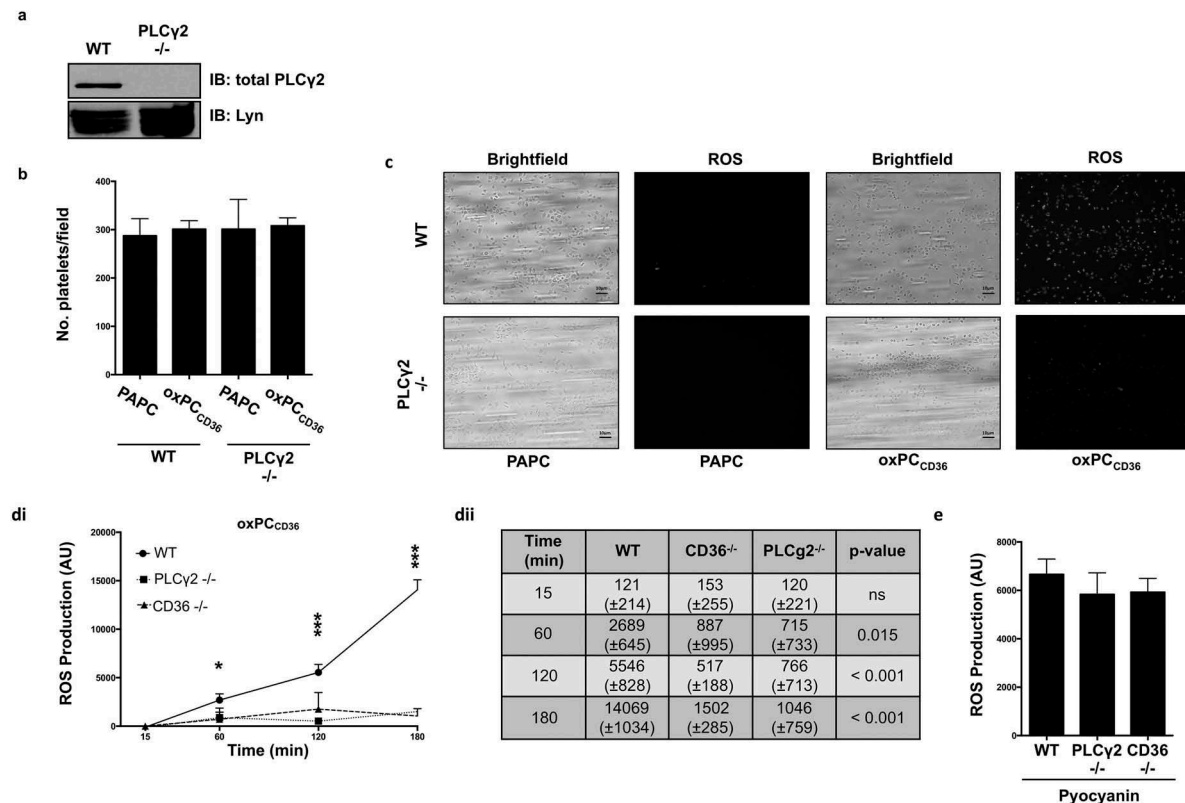
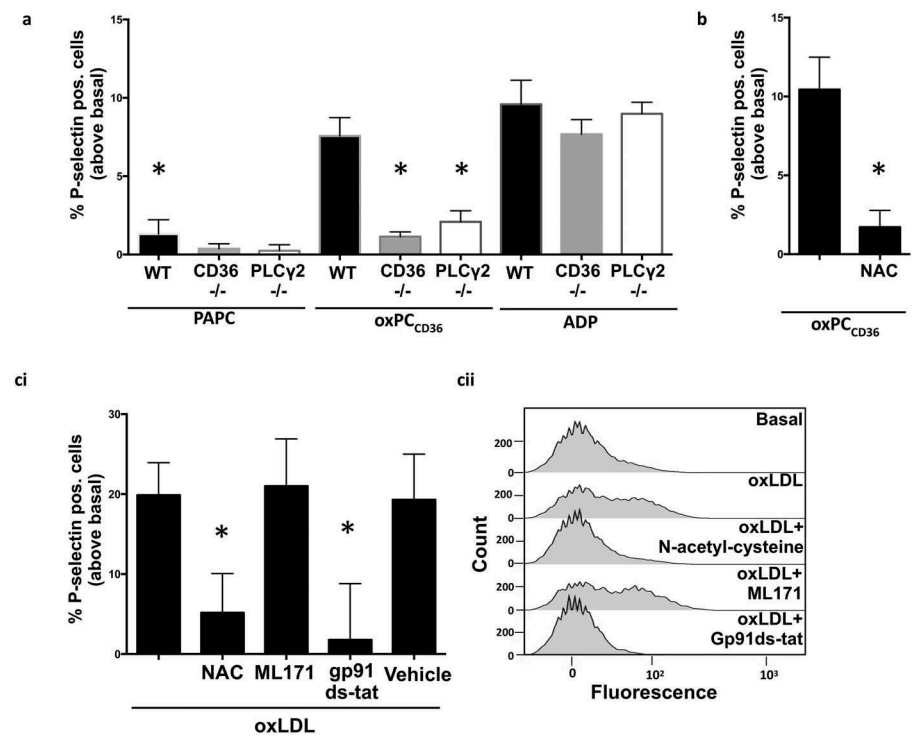


Figure 2. The oxidized phospholipid induces ROS generation in murine platelets in a CD36- and PLC $\gamma$ 2-dependent manner. (a) Washed murine platelets from WT and PLC $\gamma$ 2<sup>-/-</sup> animals were lysed and blotted for total PLC $\gamma$ 2 (1:1000) and Lyn (1:1000). (b) Wild-type and PLC $\gamma$ 2<sup>-/-</sup> murine platelets were stained with ROS-detection probe and then treated with oxPC<sub>CD36</sub> or PAPC (5  $\mu$ M). Reconstituted blood was perfused through fibrinogen-coated capillary tubes, and images of adherent platelets were then taken under bright-field microscopy and the number of adherent platelets enumerated ( $n = 3$ ). (c) As in (b), except platelets were visualized by fluorescence microscopy at 60x magnification. (di) Washed murine platelets ( $1 \times 10^8$  platelets/ml) from WT, CD36<sup>-/-</sup>, and PLC $\gamma$ 2<sup>-/-</sup> animals were incubated with the ROS-detection probe for 30 min at 37°C, then treated with oxPC<sub>CD36</sub> (25  $\mu$ M) for up to 180 min, and fluorescence measured at 520 nm ( $n = 3$ ). Data expressed as ROS production above N-acetyl-cysteine-treated platelets. \* $p < 0.05$  and \*\*\* $p < 0.001$  compared to N-acetyl-cysteine-treated platelets. (dii) Quantification of ROS production expressed as AU over time. ROS expressed as AU. (e) Washed murine platelets ( $1 \times 10^8$  platelets/ml) from WT, CD36<sup>-/-</sup>, and PLC $\gamma$ 2<sup>-/-</sup> animals were treated for 1 h with pyocyanin (250  $\mu$ M) and subsequent fluorescence measured and presented as in (d) ( $n = 3$ ).

Figure 3. OxLDL- and oxPC<sub>CD36</sub>-induced P-selectin expression is linked to ROS production. (a) PRP from WT, CD36<sup>-/-</sup>, and PLCγ2<sup>-/-</sup> animals was treated with PAPC/oxPC<sub>CD36</sub> (25 μM) or ADP (10 μM) for 60 min before fixation and flow cytometric analysis for P-selectin surface expression. \**p* < 0.05 compared to oxPC<sub>CD36</sub>-treated WT (*n* = 3). (b) As in (a), except PRP from WT mice was pretreated with N-acetyl-cysteine (5 mM) for 30 min prior to incubation with oxPC<sub>CD36</sub> (25 μM) (*n* = 3), \**p* < 0.05. (c) Human PRP pretreated with N-acetyl-cysteine (5 mM), ML171 (5 μM), and gp91ds-tat (2 μM) for 30 min and stimulated with oxLDL (50 μg/ml) for 60 min before fixation and flow cytometric analysis for P-selectin surface expression (*n* = 3). \**p* < 0.05 compared to oxLDL-treated sample. (cii) Representative histograms of human platelet flow cytometric experiments.



P-selectin was lost in CD36- and PLCγ2 deficient platelets. In contrast, the stimulatory effects of ADP were similar in all genotypes. Interestingly, oxPC<sub>CD36</sub>-induced P-selectin expression required extended incubation times and was detected after 60 min incubation. We next explored the mechanism of oxPC<sub>CD36</sub>-induced P-selectin expression. Since the expression of P-selectin in response to oxPC<sub>CD36</sub> occurred in the same time frame as that of ROS generation (Figure 2(dii)), we hypothesized that the two observations may be linked. To explore this hypothesis, we used the general ROS scavenger NAC (5 mM). Pretreatment of WT platelets with NAC caused a reduction of approximately 80% in oxPC<sub>CD36</sub>-stimulated P-selectin expression (Figure 3(b), *p* = 0.019). This is in contrast to the classical hemostatic agonists, collagen (5 μg/ml) and thrombin (0.05 U/ml), where inhibition by NAC induced approximately a 5–10% reduction in P-selectin expression and the secondary agonist ADP (10 μM) that could be reduced by approximately 25–30% (Supplementary Figure S3). These data suggest that within platelets, the sustained generation of ROS response to oxPC is required for subsequent expression of P-selectin but are less important for hemostatic agonists. To test whether cyclooxygenases (COX) are a potential source of oxPC<sub>CD36</sub>-induced ROS production, we analyzed ROS production in the presence of the pan-COX inhibitor indomethacin (10 μM) and subsequent stimulation by oxPC<sub>CD36</sub>. ROS generation was not affected by COX inhibition confirming that oxPC<sub>CD36</sub>-induced ROS generation is COX independent (Supplementary Figure S4). Having established that oxidized phospholipids induced ROS-dependent P-selectin expression in murine platelets, we examined its relevance in human platelets. Treatment of human platelets with oxLDL (50 μg/ml) led to a significant increase in P-selectin expression above basal levels (*p* < 0.001), but again required incubation times of up to 1 h. Consistent with the observation in murine platelets, P-selectin expression was blocked by NAC. Next, we

examined the potential source of ROS using pharmacological inhibitors of NOX1 (ML171; 5 μM) and NOX2 (gp91ds-tat; 2 μM), employed under conditions that blunt platelet ROS production (6). OxLDL-induced P-selectin expression was ablated in the presence of NAC (*p* = 0.034) and gp91ds-tat (*p* = 0.027), but not ML171 (Figure 3(c) and (cii)). These data confirm the previous observation and distinguish NOX2 as the likely the source of oxLDL-induced ROS production in human platelets.

## Discussion

The aim of this study was to confirm the key functional role of PLCγ2 in the transduction of atherogenic lipid stress into platelet hyperactivity. Several groups, including ours, have indicated that PLCγ2 is downstream of oxidized lipid signaling; however, the functional consequence of this signaling event remained obscure (6,7,13). Here, we demonstrate that the PLCγ2 lies downstream of CD36 ligation and show that the lipase is required for oxPC-induced ROS production and subsequent platelet activation. To the best of our knowledge, this is the first observation linking lipid stress to PLCγ2- and ROS-induced platelet activation and suggests a new role of ROS in aberrant platelet activation. Nevertheless, in the current study, we focused on PLCγ2 exclusively and cannot preclude that other isoforms may play a role in oxPC-induced ROS generation. However, given the current lack of specific PLC inhibitors, further characterization of PLC isoforms in platelets remains difficult.

Interestingly, our experiments imply that hemostatic agonists such as collagen and thrombin rely on ROS production in only a limited capacity (Supplementary Figure S3). In contrast, in oxPC<sub>CD36</sub>- and oxLDL-treated platelets, ROS appears to be an essential driver that is obligatory for platelet activation. Therefore, when ROS production is either inhibited by gp91ds-tat or scavenged by NAC, oxidized lipid-induced platelet

activation is reduced by approximately 80% in both human and murine platelets. This suggests a causal relationship between ROS and platelet activity in the context of oxidized lipid stress. Many studies have focused on the ability of oxLDL to synergize with hemostatic agonists to promote platelet hyperactivity (18–20). However, we show that prolonged incubation of platelets with oxLDL and oxPC<sub>CD36</sub> alone leads to activation and contrasts with the rapid activation induced by hemostatic agonists. This observation demonstrates a clear distinction from ROS induction by physiological agonists, where ROS are induced rapidly by both thrombin and collagen within minutes (12). Recently, the presence of oxidatively modified LDL has been demonstrated in the circulation of patients with acute coronary syndromes and stroke (21–23). In this context, our data could suggest that the prolonged exposure of platelets to oxidized lipoproteins could contribute to the sustained low-level platelet hyperactivity observed in patients (24–27). Indeed, the ROS-dependent increase in P-selectin expression may underpin an increased capacity of platelets for PSGL-1-dependent interactions with leukocytes and subsequent endothelial cell activation (28). Therefore, the current findings extend our understanding of the oxLDL–CD36 node to present a potential mechanism for a platelet-mediated prothrombotic shift, facilitated by oxidized phospholipid ligation and subsequent intracellular ROS production.

## Declaration of interest

The authors report no conflicts of interest.

## Funding

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## Supplementary material

Supplemental data for this article can be accessed [here](#).

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