Published in final edited form as:

Thromb Haemost. 2013 June ; 109(6): 1108–1119. doi:10.1160/TH12-10-0751.

Autocrine amplification of integrin α IIb β 3 activation and platelet adhesive responses by deoxyribose-1-phosphate

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Summary

Using direct injection mass spectrometry (DIMS) we discovered that deoxyribose-1-phosphate (dRP) is released by platelets upon activation. Interestingly, the addition of exogenous dRP to human platelets significantly increased platelet aggregation and integrin α IIb β 3 activation in response to thrombin. In parallel, genetically modified platelets with double genetic deletion of thymidine phosphorylase and uridine phosphorylase were characterised by reduced release of dRP, impaired aggregation and decreased integrin α IIb β 3 activation in response to thrombin. *In vitro* platelet adhesion onto fibrinogen and collagen under physiological flow conditions was potentiated by treatment of human platelets with exogenous dRP and impaired in transgenic platelets with reduced dRP release. Human and mouse platelets responded to dRP treatment with a sizeable increase in reactive oxygen species (ROS) generation and the pre-treatment with the antioxidant apocynin abolished the effect of dRP on aggregation and integrin activation. Experiments directly assessing the activation of the small G protein Rap1b and protein kinase C suggested that dRP increases the basal levels of activity of these two pivotal platelet-activating pathways in a redox-dependent manner. Taken together, we present evidence that dRP is a novel

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Conflicts of interest
None declared.

autocrine amplifier of platelet activity, which acts on platelet redox levels and modulates integrin $\alpha\text{IIb}\beta 3$.

Keywords

Platelets; reactive oxygen species; deoxyribose-1-phosphate; integrin; redox signalling

Introduction

Platelet activation is a key physiological response to tissue damage and critical step in the haemostasis process. Besides promoting the formation of multicellular aggregates (or thrombi) that help interrupting the blood loss, the surface of activated platelets hosts the coagulation cascade that leads to the generation of thrombin and ultimately the development of the fibrin clot. The tight regulation of platelet activation is therefore essential for human health. Impaired platelet activation leads to bleeding disorders (1), whereas platelet dysregulation and hyperactivity lead to thrombotic events at the basis of life-threatening cardiovascular pathologies (2, 3). Platelet activation is a complex series of events originating from the stimulation of surface receptors by biochemical signals linked to vascular damage, such as the exposure of the subendothelial matrix and the generation of thrombin in the coagulation cascade. Besides responding to their microenvironment and to the biochemical messages released by other circulating or vascular cells (4, 5), platelets also release their own rich array of extracellular signals (6). Amongst the small molecules released by platelets, thromboxane A2 (TXA2), adenosine diphosphate (ADP), epinephrine, and serotonin (5-HT) are key autocrine regulators of platelet responses. Small molecules released by platelets have been proved to regulate other important cardiovascular responses. For example, sphingosine-1-phosphate and lysophosphatidic acid have been shown to stimulate angiogenesis (7, 8), whereas the bioactive lipid platelet-activating factor (PAF) is an accepted modulator of inflammation (9).

Recently, we utilised gas-chromatography mass spectrometry (GC-MS) to identify new molecules released by platelets. The pro-angiogenic metabolite deoxyribose-1-phosphate (dRP) was detected in micromolar concentrations in stimulated platelet supernatants and the role of this metabolite in the promotion of endothelial cell migration *in vitro* and angiogenesis *in vivo* was demonstrated (10, 11). In our and other studies, intracellular formation or administration of dRP or its dephosphorylated derivative deoxyribose (dR) appeared to be linked to an increase in the generation of reactive oxygen species (ROS) (10, 12, 13). In light of the important pro-aggregatory role of ROS in platelets (14), and their proposed role in the activation of integrins (15, 16), we investigated whether platelet-derived dRP plays any autocrine role in the regulation of integrin $\alpha\text{IIb}\beta 3$ and platelet functional responses. We utilised exogenous dRP or dR and genetically modified platelets with reduced release of dRP to assess the functional relevance of these metabolites in platelet regulation. Following the identification of a potential role of dRP as positive regulator of platelet activity, the mechanism of action of this metabolite was investigated by single-cell fluorescence-based superoxide measurement, flow cytometry for the analysis of integrin

α IIB β 3 activation, pull-down assays for the investigation of Rap1b, and phosphorylation-specific immunoblot analyses.

Materials and methods

A complete list of the reagents utilised in this study is available online at www.thrombosis-online.com as Supplementary Material.

Platelet isolation

Human blood was drawn from the cubital vein of healthy volunteers under local ethic approval. Mouse blood was collected by intra-cardiac puncture following euthanasia by carbon dioxide asphyxiation. TP-/-/UP-/- mice were obtained as previously described, and the control animals (wild type, WT) were C57BL6/J from the original breeding program (17). Washed platelets were isolated as previously described (18). Acid Citrate Dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid, used at 1:7 vol/vol) was used as anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation (200 g, 15 minutes [min]) and platelets were separated from PRP by a second centrifugation step (400 g, 10 min), in the presence of prostaglandin E1 (40 ng/ml) and indomethacin (10 μ M). Platelets were resuspended in a modified Tyrode's-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 5 mM glucose, pH 7.3) at a density of 4×10^8 /ml.

Supernatant preparation and direct injection mass spectrometry

Washed platelets were resuspended at a density of 4×10^8 /ml. Where indicated human and mouse platelets were stimulated with human thrombin (0.1 and 1 unit/ml, respectively) for 5 min at 37°C using a 490D aggregometer (Chrono-Log Corporation, Havertown, PA, USA). Human plasma, resting platelet supernatants and thrombin-stimulated supernatants were filtered using Vivaspın 15R Hydrosart columns (2,000 Da cut-off) and analysed for 60 seconds (sec) by direct infusion mass spectrometry (DIMS) using an electrospray LTQ-Orbitrap hybrid mass spectrometer operating in negative ion mode in the mass range 100-1,000 Da (ThermoFisher Scientific, Bremen, Germany). All scans were averaged, background was subtracted and the ion count (m/z 213.017) was used as semi-quantitative estimation of dRP levels. Accurate method descriptions for DIMS have been published previously (11, 19).

Platelet aggregation

Washed human platelets were stimulated with human thrombin (0.01, 0.05, and 0.1 units/ml), or the thromboxane A₂ mimetic U46619 (100 nM), fibrillar collagen (native collagen fibrils type I from equine tendons, 10 μ g/ml), whereas washed mouse platelets were stimulated with human thrombin (0.5 units/ml). Where indicated platelets were pre-incubated for 5 min with dRP (10, 40, and 200 μ M), dR (200 μ M), or apocynin (0.5 mM). Aggregation was measured for 4 min at 37°C using a 490D aggregometer (Chrono-Log Corporation).

Analysis of integrin $\alpha\text{IIb}\beta 3$ activation by flow cytometry

Washed human platelets ($4 \times 10^7/\text{ml}$) were incubated for 5 min with 0.5% vol/vol Fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody, whereas washed mouse platelets were incubated for 5 min with 5% vol/vol FITC-conjugated JON/A antibody. Where indicated inhibitors and other pharmacological tools were co-incubated with the antibodies. Integrin activation was obtained with human thrombin for 5 min in static conditions (0.05 and 0.1 unit/ml for human platelets, 0.5 unit/ml for mouse platelets). Following 1 in 10 dilution in phosphate buffer saline (PBS, pH 7.4: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4), platelets were then analysed by flow cytometry for FITC staining using FACSCantoII (Becton Dickinson, Oxford, UK) and the results were analysed using FACSDiva software (Becton Dickinson).

In vitro platelet adhesion under flow

Platelet adhesion under flow was analysed in whole blood using a Bioflux 200 system (Fluxion, San Francisco, CA, USA). Flow microchannels were coated with 0.1 mg/ml fibrinogen or 0.1 mg/ml fibrillar collagen I or 1 mg/ml collagen I, before blocking with 1% bovine serum albumin (BSA) and washing with modified Tyrode's buffer. For experiments on human blood, PRP was isolated from anticoagulated blood (citrate) by centrifugation (200 g, 15 min) and incubated for 1 hour with Calcein BlueTM (5 $\mu\text{g}/\text{ml}$) at 37°C. PRP was then added to the red blood cell fraction and where indicated dRP was added (200 μM). Platelet adhesion was visualised by fluorescence microscopy and video clips were obtained by collating pictures taken every 10 sec (real-time analysis). For experiment with mouse blood, whole blood was used without isolation of PRP and images were taken after 5 min by flushing away non-adhering blood cells with modified Tyrode's buffer before phase contrast microscopy (endpoint analysis). Platelet adhesion under flow conditions was tested on collagen I (collagen monomers from calf skin, shear rate = $1,000 \text{ sec}^{-1}$), fibrillar collagen I (native collagen fibrils type I from equine tendons, shear rate = $1,000 \text{ sec}^{-1}$), and human fibrinogen (shear rate = 200 sec^{-1}).

Intracellular ROS generation assay

The generation of ROS in platelets was investigated using a dihydroethidium (DHE)-based single cell fluorescence assay, with excitation provided by the 543 nm line of the helium-neon laser line and emitted fluorescence collected $>560 \text{ nm}$ (20). Platelets adhering to human fibrinogen on 22 mm glass cover slips were treated with DHE (10 μM). Platelets were then activated with human thrombin (0.1 and 1 unit/ml for human and mouse platelets, respectively) or treated with dRP (200 μM). The rate of DHE oxidation is proportional to intracellular ROS generation and was measured as fluorescence increase (relative unit/second). Images were obtained using a Zeiss 510 LSM confocal microscope equipped with a 40X oil-immersion lens.

RAP1B pull-down activation assay

Washed platelets ($4 \times 10^8/\text{ml}$) were stimulated with 0.05 unit/ml human thrombin for 5 min (no stirring) without or with previous dRP and/or apocynin incubation (as indicated). Resting and stimulated platelets were lysed with modified radioimmuno-precipitation assay

(RIPA) buffer (final concentration: 1% Triton X-100, 1% sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 50 mM Tris, pH 7.4) containing EDTA-free protease. Positive and negative controls were obtained by treatment of the resting lysates with EDTA (10 mM) followed by GTP γ S or GDP (0.1 mM and 1 mM, respectively). Activated Rap1b was selectively separated from the lysates using a glutathione S-transferase (GST)-Ral guanine nucleotide dissociation stimulator (RalGDS)-Rap1 binding domain (RBD) recombinant fusion protein and glutathione resin (Thermo Scientific). Following dissociation from glutathione resin in the presence of β -mercaptoethanol, active Rap1b was determined by immunoblot.

Determination of PKC activity by immunoblot

As described previously (21), platelets were resuspended at a final density of 4×10^8 /ml and treated with 1 mM ethylene glycol tetraacetic acid (EGTA) immediately before stimulation. Platelet stimulation was obtained with 0.1 unit/ml thrombin for 5 min under stirring conditions using a 490D aggregometer (Chrono-Log Corporation). Cell lysis was achieved with radioimmuno-precipitation assay buffer (RIPA) (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4) containing protease and phosphatase inhibitors. Proteins were resolved by SDS/PAGE and a specific antibody that recognises PKC phosphorylation sites in their phosphorylated form was used for immunoblotting. Therefore, the number and the intensity of the bands in the immunoblots provide an estimation of the activation levels of classical PKC isoforms in the different conditions.

Statistical analysis

Data are expressed throughout as mean $\pm$ SEM. The results were analysed by t-test (for comparisons of two groups), one-way ANOVA with Bonferroni post-test (for multiple comparisons), or two-way ANOVA with Bonferroni post-test (for time courses).

Results

In a previous study we reported the stimulation-dependent release of dRP by platelets 10. In this study, we widened the mass spectrometry analysis to the dephosphorylated derivative dR, which has previously been reported to stimulate integrin activation in endothelial cells and has therefore the potential to play a role in regulating platelet functional responses (22). The detection of specific ions for dR and dRP by direct injection mass spectrometry (DIMS) (► Figure 1 B) allowed semiquantitative determination of the levels of these metabolites in human platelet supernatants and in human plasma. As shown in ► Figure 1 A, dRP appears at significantly higher concentration in the supernatant of thrombin-stimulated platelets compared to resting platelets and plasma (one-way ANOVA with Bonferroni post-test, $n=5$). Based upon the sensitivity of the experimental procedure and equipment utilised, the concentration of dRP in platelet supernatants is estimated to be in the micromolar range (50-400 μ M). For dR, the situation was substantially different, with no significant release by human platelets upon stimulation and plasma levels higher than those in platelet supernatants (although not significantly, one-way ANOVA with Bonferroni post-test, $n=6$).

Next we assessed whether either dRP or dR can modulate human platelet aggregation in response to the physiological agonist thrombin. While dRP on its own did not stimulate any aggregatory response, platelet aggregation in response 0.05 unit/ml thrombin was potentiated by this metabolite in a concentration-dependent manner (200 μ M dRP > 40 μ M dRP > 10 μ M dRP) (► Figure 2 A). Interestingly, pretreatment with dRP promoted platelet response to 0.01 unit/ml thrombin, which usually elicits only negligible aggregation (% aggregation: 18.6 ± 1.5 and 4.4 ± 0.9 , $p < 0.01$, $n = 4$; representative aggregation traces on ► Figure 2 B), whereas the effect of dRP was negligible on the high thrombin concentration (0.1 unit/ml) (► Figure 2 B). In contrast, pre-incubation with 200 μ M dR did not affect platelet aggregation in response to 0.01, 0.05, or 0.1 unit/ml thrombin (► Figure 2 C). In addition, pre-incubation with dRP potentiated human platelet aggregation in response to the TXA2 analog U46619 (100 nM, see Suppl. Figure 1B), but not collagen (10 μ g/ml, see Suppl. Figure 1A, available online at www.thrombosis-online.com), which indicated some degree of agonist selectivity of the activity of dRP. Since platelet aggregation is known to depend on integrin α IIB β 3, the activation of this integrin in response to thrombin was directly investigated by flow cytometry using an active-state selective antibody in human and mouse platelets. In agreement with aggregation data, 200 μ M dRP significantly potentiated human integrin α IIB β 3 activation in response to a submaximal thrombin concentration (0.05 unit/ml) but was ineffective on a high thrombin concentration (0.1 unit/ml) (► Figure 2 D). Also in agreement with aggregation data, dR did not potentiate the response to either 0.05 or 0.1 unit/ml thrombin (► Figure 2 D). The population of washed platelets analysed by flow cytometry is described in the side scattering (SSC) versus forward scattering (FSC) dot plot shown in see Suppl. Figure 2A (available online at www.thrombosis-online.com). A representative example of the effect of dRP on thrombin-dependent α IIB β 3 activation analysed by PAC-1 immunostaining is shown in Suppl. Figure 2B (available online at www.thrombosis-online.com). The potentiation of integrin α IIB β 3 activation by dRP was also confirmed in mouse platelets (see Suppl. Figure 2C, available online at www.thrombosis-online.com).

Besides the effect of increased dRP levels on human platelets by addition of exogenous dRP, we tested how the reduction of endogenous dRP release affects platelet activity in a mouse model characterised by genetic deletion of thymidine phosphorylase (TP) and uridine phosphorylase (UP) (17), two major enzymes for the production of dRP in platelets (10). The genetic silencing of TP and UP in transgenic mouse platelets was assessed by immunoblot (see Suppl. Figure 3A, available online at www.thrombosis-online.com) and the reduced release of dRP by thrombin-stimulated platelets was confirmed by DIMS (see Suppl. Figure 3B, available online at www.thrombosis-online.com). TP^{-/-} UP^{-/-} platelets displayed reduced aggregation (% aggregation: 29.4 ± 3.1 vs 49.9 ± 4.4 , $p < 0.05$, $n = 5$; representative aggregation traces in ► Figure 3 A) and reduced integrin α IIB β 3 activation (► Figure 3 B) in response to 0.5 unit/ml thrombin compared to WT platelets. In both cases, full responses were rescued by addition of 200 μ M exogenous dRP to TP^{-/-} UP^{-/-} platelets (► Figure 3 A and B).

The potential of platelet release of dRP in the regulation of thrombus formation in whole blood was tested *in vitro* under flow conditions. Human platelet adhesion onto either fibrinogen-coated (► Figure 4 A and Suppl. Movie 1, available online at www.thrombosis-online.com)

www.thrombosis-online.com; shear rate 200 sec⁻¹) or monomeric collagen I-coated surfaces (► Figure 4 B and Suppl. Movie 2, available online at www.thrombosis-online.com; shear rate 1,000 sec⁻¹) was significantly increased by the addition of 200 µM dRP. Conversely, whole blood from TP^{-/-} UP^{-/-} animals was characterised by reduced platelet adhesion onto both fibrinogen (► Figure 4 C) and collagen I (► Figure 4 D). In the presence of added exogenous dRP (200 µM) transgenic and WT platelets displayed similar adhesion to both fibrinogen (► Figure 4 E) and collagen I (► Figure 4 F). Notably, thrombus formation on fibrillar collagen I (1,000 sec⁻¹) was not affected by exogenous dRP (see Suppl. Figure 4A, available online at www.thrombosis-online.com) or genetic ablation of TP and UP (see Suppl. Figure 4B, available online at www.thrombosis-online.com).

In order to understand the mechanism of action of dRP and because of ours and other's previous results suggesting that dRP and its derivatives promote intracellular ROS generation (10, 12, 23), we investigated the effect of dRP and dR on intracellular ROS levels in platelets using single-cell dihydroethidium (DHE)-based fluorescence imaging. Interestingly, and in line with the functional results in ► Figure 2 C and D showing no platelet activity potentiation, dR did not stimulate any significant increase in ROS generation over the control (vehicle solution), whereas dRP did (p<0.01, two-way ANOVA) (► Figure 5 A). The generation of ROS induced by dRP was abolished by the antioxidant apocynin in a concentration-dependent manner (► Figure 5 B, IC₅₀ = 87µM). Notably we and others have recently shown apocynin to inhibit ROS generation in platelets by acting as an antioxidant besides inhibiting NADPH oxidases (24, 25). In order to understand whether the inhibition of NADPH oxidases or the antioxidant activity of apocynin were crucial for the inhibition of dRP-dependent ROS accumulation, we utilised the NADPH oxidase-specific inhibitor 2-acetylphenothiazine (2APT) and the ROS scavenger N-acetylcysteine (NAC). As expected, 1 mM NAC completely abolished the accumulation of ROS, while surprisingly 10µM 2APT (which has been shown to inhibit platelet NADPH oxidases [26]) significantly but only partially inhibited the accumulation of ROS (p<0.01, two-way ANOVA) (► Figure 5 C).

The redox-dependence of the effect of dRP on thrombin-dependent platelet responses was assessed using apocynin, which has been shown to significantly impair ROS generation in platelets (15). Indeed, 0.5 mM apocynin abolished the potentiation of thrombin-dependent human platelet aggregation (► Figure 6 A) and integrin αIIbβ3 activation (► Figure 6 B) induced by 200 µM dRP. The dependence of aggregation on ROS generation was further confirmed by experiments with the ROS scavenger NAC, which strongly inhibited platelet aggregation induced by 0.1 unit/ ml thrombin when pre-incubated at a concentration of 1 mM (78.4 ± 5.8 and 11.8 ± 4.0, p<0.01, n=3) and completely abolished it at a concentration of 10 mM (see Suppl. Figure 5A, available online at www.thrombosis-online.com). On the other hand, the potentiation of thrombin-induced platelet aggregation by dRP does not depend on increased release of ADP, as it was not affected by platelet incubation with the ADP scavenger apyrase (see Suppl. Figure 5B, available online at www.thrombosis-online.com).

The molecular mechanism of action of dRP downstream of ROS induction was investigated by analysing Rap1b (pull-down assay) and PKC (phosphor-specific immunoblot) activation

in human platelets. Interestingly, the treatment of platelets with dRP resulted in a detectable increase in the level of Rap1b activation both in the presence (► Figure 7 A) and absence (► Figure 7 B) of thrombin-stimulation. In parallel with α IIB β 3 activation data, apocynin (0.5 mM) abolished the potentiation induced by dRP in Rap1b activation levels in the presence and absence of thrombin stimulation (► Figure 7 A and B, respectively). Finally, the effect of dRP on PKC activity was studied using an antibody that recognises the phosphorylated state of the phosphorylation target sites for classical PKC isoforms on different substrates (21). The pretreatment of platelets with dRP (200 μ M) resulted in a detectable increase in the basal level of activity of PKC (visualised as an increase in number and intensity of the immunobands in lane 2 compared to lane 1, ► Figure 7 C). Apocynin inhibited the stimulatory effect of dRP on PKC activity (lanes 3 and 4, ► Figure 7 C). Notably, the pre-incubation of platelets with dRP did not seem to increase the levels of PKC activation above the levels reached by thrombin alone (lanes 5 and 6, ► Figure 7 C). Similarly, phospho-specific immunoblots for the PKC substrates pleckstrin and myosin light chain (MLC) revealed potentiation of PKC-dependent phosphorylation in platelets treated with 200 μ M dRP (see Suppl. Figure 6A). Interestingly, the activity of the kinases Src, p38-mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERKs) was also stimulated above basal levels by treatment with 200 μ M dRP (see Suppl. Figure 6B-D, available online at www.thrombosis-online.com).

Discussion

The investigation of platelet regulation is of central importance for the understanding of thrombotic and bleeding disorders and for the design of appropriate clinical interventions. In this study we have discovered that dRP is a novel autocrine regulator of platelets and we have characterised the basics of its molecular mechanism of action. We previously reported the identification of dRP in the releasate of human and mouse platelets upon activation by physiological stimuli using a mass spectrometry-based metabolomics approach (10). Platelet-derived dRP was shown to promote endothelial cell motility in a ROS-dependent manner and to stimulate angiogenesis *in vivo* (10). The pro-angiogenic activity of dRP and its de-phosphorylated derivative dR in mammalian systems had already been reported (27, 28). Since the pro-angiogenic activity of dR has been associated with the stimulation of integrin outside-in signalling (22) and the generation of ROS (12), here we investigated whether platelet activity, which depends on integrin activation and is modulated by ROS, is also modulated by dR or its precursor dRP in an autocrine manner.

The investigation of the presence of dRP and dR in platelet supernatants and plasma by mass spectrometry suggested that the former rather than the latter could play some modulatory role on platelets in physiological conditions. In fact, similarly to other autocrine modulators of platelets (29,30), dRP appeared to be released in a stimulation-dependent manner, and its concentration in activated platelet supernatants appeared to be several times higher compared to plasma. This suggests that significantly higher local concentrations of dRP can be found in vascular microenvironments characterised by platelet activation, where this metabolite might be able to function as platelet activation and haemostasis amplifier. The concentration of dRP released by platelets was estimated in the high micromolar, although a truly quantitative method was not utilised. Based on “ion count” and instrument sensitivity

we estimated the concentration range between 80 and 400 μM . This would be significantly higher than the levels of other platelet released metabolites, such as adenosine di-phosphate (7-10 μM) and adenosine tri-phosphate (15-20 μM) (31). This can be due to high levels of expression of the nucleoside phosphorylases responsible for the generation of dRP or high efficiency of the transport systems responsible for accumulating dRP in platelet cytoplasm and granules. In contrast, dR appeared to be released at negligible concentration by platelets (either resting or activated), and the concentration in platelet supernatants was comparable or lower than the concentration in plasma. These observations are incompatible with a physiological role of dR as a local regulator of platelets or haemostasis.

Our functional data clearly suggested a potentiatory effect of dRP but not dR on platelet aggregation. The addition of exogenous dRP resulted in significantly increased human platelet aggregation in response to low thrombin concentration (0.05 unit/ml) and to U46619, whereas the aggregation in response to fibrillar collagen or high thrombin concentration (0.1 unit/ml) was not significantly affected. These results might suggest that in our experimental conditions collagen and high thrombin concentrations stimulate maximal platelet activation, which could not be modulated by the addition of exogenous dRP. On the other hand, the thromboxane A2 analog U46619 and low thrombin concentrations induce submaximal platelet activation, which could be significantly affected by the addition of exogenous dRP in our experiments. Accordingly, the ablation of dRP in $\text{TP}^{-/-}$ $\text{UP}^{-/-}$ platelets corresponded to a reduction of aggregation, which suggested that this metabolite might play a relevant role in the autocrine regulation of platelets in physiological conditions. In this respect, it seems particularly relevant that in the presence of dRP extremely low thrombin concentrations (0.01 unit/ml) normally unable to elicit platelet activation induced a detectable aggregatory response. This might warrant further research on whether dRP plays a role in the determination of platelet hyperaggregability in inflammatory syndromes and cancer, which are characterised by the upregulation of dRP-generating enzymes (32-35). Addition or depletion of dRP were also shown to affect the activation levels of integrin $\alpha\text{IIb}\beta 3$, which is known to play a key role in the aggregation process by mediating fibrinogen-dependent platelet-platelet interactions (36). In agreement with the aggregation results, dRP appeared to potentiate the activation of integrin $\alpha\text{IIb}\beta 3$ at low thrombin concentration but not at high thrombin concentration, whereas dR was not able to affect the activation level of this integrin at any thrombin concentration. Although our data on dR are in disagreement with a previous study in endothelial cells showing a potentiatory role for this metabolite on integrin activity (22), the difference in cell type (platelet versus endothelial cells), integrin dimer analysed ($\alpha\text{IIb}\beta 3$ vs $\alpha 5\beta 1$ and $\alpha \nu \beta 3$) and signalling event monitored (integrin activation versus outside-in signalling and focal adhesion formation) might explain this discrepancy.

Besides homotypic aggregation mediated by fibrinogen (i.e. platelet-to-platelet adhesion), integrin $\alpha\text{IIb}\beta 3$ is also critical for the interaction of platelets with extracellular matrix proteins (e.g. collagen, which is mediated by von Willebrand factor [37]), and with other fibrinogen-binding cells, such as endothelial and tumour cells (38). Hence, we utilised a whole blood model to assess whether dRP affects the adhesion of platelets to collagen- and fibrinogen-coated surfaces under flow conditions. As shown previously (39), the adhesion of platelets on fibrinogen is efficient at low and moderate shear rate, possibly due to the

reported low resistance to tensile stress of the bond between fibrinogen and integrin $\alpha\text{IIb}\beta 3$ (40). For this reason, adhesion to fibrinogen was tested at a shear stress of 200 sec^{-1} , at which exogenous dRP addition significantly increased the adhesion of human platelets while endogenous dRP depletion significantly reduced the adhesion of transgenic $\text{TP}^{-/-} \text{UP}^{-/-}$ mouse platelets. These results correlate well with our flow cytometry results suggesting that dRP facilitates the activation of integrin $\alpha\text{IIb}\beta 3$, which is the main receptor for fibrinogen on platelets. As suggested by previous studies (41), the adhesion on collagen was tested at shear rate $1,000 \text{ sec}^{-1}$ on either monomeric collagen I or fibrillar collagen I (also known as Horm collagen), which led to substantially different results. Similarly to results obtained with fibrinogen, exogenous dRP addition significantly increased whereas endogenous dRP depletion significantly reduced the adhesion of platelets on monomeric collagen I, whereas the adhesion on fibrillar collagen I was not affected by dRP. This correlates with our results on fibrillar collagen-induced platelet aggregation (i.e. absence of dRP effect shown in Suppl. Figure 1A, available online at www.thrombosis-online.com) and might reflect the fact that effect of dRP cannot influence the high levels of platelet activation induced by fibrillar collagen I compared to monomeric collagen I (42). Alternatively, the absence of dRP effect in these experiments might suggest that in whole blood under flow conditions surface adhesion proteins other than integrin $\alpha\text{IIb}\beta 3$ are responsible for the binding of fibrillar collagen (e.g. integrin $\alpha 2\beta 1$, glycoprotein VI, or glycoprotein Ib) (41).

Previous studies have shown that dRP (12) and its dephosphorylated derivative dR (13, 43) induce intracellular ROS generation. In view of the role proposed for ROS in platelet activation (14, 24), we investigated whether dRP is also associated with an increase in ROS generation in platelets. Using a dihydroethidium-based single cell imaging approach we proved that exogenous dRP addition to human platelets induces an increase in the intracellular generation of ROS. Although previous studies suggested that the generation of ROS induced by dRP and dR was due to direct protein glycation (13), the source of intracellular ROS in dRP-treated platelets remains undefined. As suggested by the partial effect of the specific NADPH oxidase inhibitor 2APT compared to the complete inhibition observed with the ROS scavenger NAC (► Figure 5 C), NADPH oxidase play a role in the dRP-dependent accumulation of ROS although other sources also contribute. In our hands, dR did not stimulate any significant increase in intracellular ROS generation in platelets. The apparent discrepancy regarding dR between this and previous studies might be due to different concentrations utilised ($200 \mu\text{M}$ vs $10\text{-}50 \text{ mM}$), different experimental approach (measurement of intracellular ROS generation versus peroxide generation), and different cell types (platelets vs pancreatic or monocytic cells) (13, 43). Also because of the different concentrations of these metabolites in platelet releasates, dRP appears to play a relevant pro-activatory role on platelet regulation whereas dR does not. In agreement with previous studies (14, 44, 45), besides abolishing dRP-dependent platelet potentiation, the pharmacological reduction of ROS generation in our experiments impaired normal platelet functional responses and signalling (i.e. aggregation in ► Figure 6 A and in Suppl. Figure 5A, integrin $\alpha\text{IIb}\beta 3$ activation in ► Figure 6 B, and Rap1b activation in ► Figure 7 A), thus confirming a relevant regulatory role for these highly reactive molecules in platelet physiology. It is therefore not surprising that dRP and other molecules able to modulate intracellular ROS generation have a powerful regulatory role on platelets and haemostasis.

Although the potentiating effects of ROS on platelet activity were described some time ago (45), the molecular pathway responsible for it is not fully elucidated. The regulation of integrin $\alpha\text{IIb}\beta 3$ has been proposed as target of the ROS-dependent regulation of platelets. Either the redox balance of sulfhydryl groups (46) or the tyrosine phosphorylation (15) of the $\beta 3$ subunit have been proposed to modulate the activation of integrin $\alpha\text{IIb}\beta 3$. Since Rap1b is known to play a crucial role in the activation of integrin $\alpha\text{IIb}\beta 3$ in platelets (47), in this project we tested the effect of exogenous dRP on the activation of this small GTPase. We found that the addition of exogenous dRP to human platelets directly induced intermediate levels of Rap1b activation. Therefore, we propose that the dRP directly activates Rap1b, which facilitates integrin $\alpha\text{IIb}\beta 3$ activation and potentiates agonist-dependent platelet activation, aggregation, and adhesion. Interestingly, the direct activation of Rap1b by dRP did not correspond to the direct activation of integrin $\alpha\text{IIb}\beta 3$ by this metabolite, perhaps suggesting that the regulation of this integrin is a multifactorial process that requires the presence of a physiological agonist. Finally, we have shown that the incubation of platelets with exogenous dRP led to an increase in the basal activity of PKC in the absence of thrombin, which is known to be involved in the activation of rap1b (48). The inability to detect dRP-dependent increase in PKC activity in the presence of thrombin is probably due to the lack of sensitivity of our experimental approach (phospho-specific immunoblot) in conditions of high PKC activity. Because of the central role of PKC in platelet regulation and the impossibility of inhibiting this enzyme without completely obliterating platelet activation (49), it is impossible at this stage to understand whether the activation of PKC by dRP-induced ROS is indeed responsible for the potentiation of integrin $\alpha\text{IIb}\beta 3$ and platelet activation that we observed in our experiments. It is also impossible to elucidate whether dRP-dependent PKC activation is the cause of Rap1b activation or whether Rap1b and PKC activation are independently affecting $\alpha\text{IIb}\beta 3$, which has also been suggested previously (50). The increase in basal activity of the kinases Src, p38-MAPK and ERKs are also original observations that might explain the potentiation of platelet responses, although they are likely to be a consequence of the stimulation of PKC rather than an independent effect of dRP.

In summary, we present novel *in vitro* evidence that dRP is an autocrine amplifier of platelet activation, aggregation, and adhesion. Although at this stage it is impossible to determine whether dRP acts in a receptor-mediated manner or even to understand whether it crosses the plasma membrane and performs its activity in the cytoplasm, the regulation of platelet by dRP appears to be mediated by ROS generation, which leads to the stimulation of increased levels of PKC and Rap1b activation and results in the potentiation of integrin $\alpha\text{IIb}\beta 3$ activation levels. Further *in vivo* and clinical studies are required to determine the relevance of endogenous dRP in the modulation of platelets and haemostasis in physiological and/or pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Mr Adrian Rogers (University of Bath) and Dr Andrew Hibbert (Royal Veterinary College) for their help with live platelet imaging. They also would like to acknowledge the kind help of Dr Ramon Martí, Dr Javier Torres-Torronteras and Dr Antoni L. Andreu (Hospital Universitari Vall d'Hebron, Barcelona, Spain) for breeding and importation of transgenic mice.

Financial support:

The work with transgenic animals was sponsored by the Royal Society (RG090131), whereas the studies on human platelets were supported by the BBSRC (BB/J002690/1).

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What is known about this topic?

- Mammalian platelets express high levels of enzymes able to generate deoxyribose-1-phosphate (dRP), with thymidine phosphorylase (initially isolated from platelets as platelet-derived endothelial cell growth factor or PD-ECGF), uridine phosphorylase and purine nucleoside phosphorylase all expressed in human platelets.
- Our previous work described the release of micromolar concentrations of dRP upon platelet activation.
- Except for the effect of dRP on endothelial cell motility and angiogenesis that we and others have described, very little is known about the effect of this molecule on platelet activity and the physiological role of its release by platelets.

What does this paper add?

- Here, for the first time, we show that exogenous and endogenous (i.e. stimulation-dependently released) dRP positively modulates platelet activation, leading to increased platelet aggregation in response to thrombin (but not collagen) and increased thrombus formation in whole blood under flow conditions.
- The exposure of human and mouse platelets to dRP leads to increased reactive oxygen species generation, which potentiates PKC, Rap1b and integrin $\alpha\text{IIb}\beta 3$ activation in a redox-dependent manner.
- In summary, this manuscript describes dRP as a novel physiological modulator of platelet activity and identifies the enzymes which generate it as a potential drug target for cardiovascular applications.

Figure 1. Detection of dRP and dR by direct infusion mass spectrometer (DIMS) of platelet supernatants

Human platelet stimulation was obtained with 0.1 unit/ml thrombin for 5 min under stirring (700 rpm). The supernatant of thrombin-stimulated platelets (Plt. (thr.)), the supernatant of resting platelets (Plt. (rest.)), and human plasma from the same donors were filtered using Vivaspin 15R Hydrosart columns (2,000 Da cut-off). Samples were infused for one minute at a flow rate of 5 μ l/min with data acquired in the mass range 100-1,000 Da. The hybrid LTQ-Orbitrap mass spectrometer was tuned for detection of dRP and operated with a mass resolution of 100,000 (FWHM at mass 400 Da). A) All scans were averaged, and species-

specific ion counts were employed for semi-quantification of dRP and dR concentration. The data are absolute counts for dR- and dRP-specific ions (m/z 213.0165 and 133.0505, respectively) and are presented as mean $\pm$ SEM from five independent samples. Statistical significance was tested by ANOVA with Bonferroni post-test (* $p < 0.05$). B) Complete ionisation profile of platelet supernatants with the m/z values for high abundance peaks is shown, with the inserts showing the region of the spectra specific for dR and dRP around m/z 133.05 and 213.00, respectively (dR; $C_5H_{10}O_4$; $[M-H]^-$; m/z 133.0505; mass error = -0.8 ppm; dRP; $C_5H_{11}O_7P$; $[M-H]^-$; m/z 213.0165; mass error = -0.8 ppm).

Figure 2. Potentiation of human platelet aggregation and integrin $\alpha\text{IIb}\beta 3$ by exogenous dRP Washed platelets were pre-incubated for 5 min with vehicle solution (modified Tyrode's buffer) or the indicated concentrations of dRP (A, B, and D) or dR (C and D). Platelet activation was obtained with the indicated concentrations of thrombin (0.01, 0.05 and 0.1 unit/ml). Aggregation was monitored by turbidimetry for 4 min at 37°C under stirring (700 rpm) (A, B, and C), whereas integrin $\alpha\text{IIb}\beta 3$ activation in response to 0.05 unit/ml thrombin was monitored without stirring by flow cytometry using an activation-dependent FITC-conjugated antibody (Pac-1) (D). The aggregation traces are representative of three or more independent experiments, whereas the fluorescence values for the flow cytometry experiments are fold-increase ratios over basal (no thrombin) and are expressed as mean $\pm$ SEM from four independent experiments. Statistical significance was tested by one-way ANOVA with Bonferroni post-test (* $p < 0.05$).

Figure 3. Potentiation of mouse platelet aggregation and integrin α IIb β 3 by endogenous dRP Washed platelets from wild type (WT) and TP^{-/-} UP^{-/-} mice were stimulated with 0.5 unit/ml thrombin in the presence or absence of 200 μ M dRP. Aggregation was monitored by turbidimetry for 4 min at 37°C under stirring (700 rpm) (A), whereas integrin α IIb β 3 activation in response to 0.5 unit/ml thrombin was monitored without stirring by flow cytometry using an activation-dependent FITC-conjugated antibody (JON/A) (B). Representative aggregation traces are shown (n=8), whereas the fluorescence values for the flow cytometry experiments are fold-increase ratios over basal (no thrombin) and are expressed as mean $\pm$ SEM (n=6). Statistical significance was tested by one-way ANOVA with Bonferroni post-test (* p<0.05).

Figure 4. Endogenous and exogenous dRP facilitate platelet adhesion under flow
Human platelet-rich plasma (PRP) was isolated from anticoagulated blood (citrate) and incubated for 1 hour with Calcein Blue™ (5 µg/ml) at 37°C. After reconstitution of whole blood by mixing labelled PRP and the red blood cell fraction, adhesion to fibrinogen (A) and collagen I (B) (200 sec⁻¹ and 1,000 sec⁻¹, respectively) were tested in the absence or presence of exogenous dRP (200 µM). For mouse experiments, whole blood from wild type (WT) or TP^{-/-} UP^{-/-} animals was also tested for adhesion to fibrinogen (C and E) and collagen I (D and F) at shear rate 200 sec⁻¹ and 1,000 sec⁻¹, respectively. Exogenous dRP (200 µM) was added to mouse whole blood where indicated (E and F). Adhered platelets were visualised after 5 min of flow by epifluorescence microscopy (human) or by phase contrast microscopy (mouse). Representative pictures from three independent experiments are shown and values of % surface area coverage (mean ± SEM) are presented. Statistical analysis was performed by t-test (* p < 0.05).

Figure 5. Endogenous and exogenous dRP increase the intracellular generation of ROS in platelets

The rate of intracellular ROS generation in platelets adhering to fibrinogen-coated surface was measured using dihydroethidium (DHE, 10 μM) with excitation provided by the 543 nm line of the helium-neon laser line and emitted fluorescence collected >560 nm. Traces shown in (A) indicate the rates of ROS production in human platelets before and after addition of exogenous dRP and dR (200 μM) compared to control (vehicle solution = Tyrode's buffer). The effects 200 μM dRP with or without pre-treatment with apocynin concentrations ranging from 50 to 500 μM are shown in (B). ROS production in response to dRP (200 μM) was also measured in the presence of 1mM NAC or 10 μM 2APTt (C). Both compounds significantly inhibit ROS accumulation although the inhibition by 2APT is only partial (two-way ANOVA, $p < 0.01$). The traces presented here are the accumulation rate of ROS measured as DHE-dependent fluorescence staining of single platelets in live cell confocal microscopy experiments ($n=5$).

Figure 6. The antioxidant and NADPH oxidase inhibitor apocynin abolishes dRP-dependent potentiation of platelet aggregation and integrin α IIb β 3 activation
A) Washed human platelets were incubated for 5 min with or without 0.5 mM apocynin, then treated for further 5 min with 200 μ M dRP (or vehicle solution) before stimulation with 0.05 unit/ml thrombin. Aggregation was measured by turbidimetry for 4 min at 37°C under stirring (700 rpm). Traces presented here are representative of at least three independent experiments. B) Integrin α IIb β 3 activation by 0.05 unit/ml thrombin in the presence or absence of 200 μ M dRP was monitored with or without 0.5 mM apocynin pre-incubation by flow cytometry using an activation-dependent FITC-conjugated antibody (Pac-1). The fluorescence values for the flow cytometry experiments are fold-increase ratios over basal (no thrombin) and are expressed as mean $\pm$ SEM (n=5). Statistical significance was tested by one-way ANOVA with Bonferroni post-test (* p<0.05).

Figure 7. Exogenous dRP increases Rap1b and PKC activation in human platelets

Rap1b activation was measured in a pull down assay using a GST-RalGDS-RBD recombinant protein. Washed human platelets were pre-incubated for 5 min with or without 0.5 mM apocynin, then treated for further 5 min with 200 μ M dRP (or vehicle solution) before stimulation with 0.05 unit/ml thrombin (A) or mock stimulation with vehicle solution (A and B) without stirring for 5 min. Following platelet lysis, active Rap1b was captured by interaction with GST-RalGDS-RBD and detected by specific immunoblotting (Rap1b-GTP). 20 μ l of total cell lysates for each condition were also loaded as a control (Total Rap1b). The immunoblot shown here are representative of at least three independent experiments. C) The activity of PKC was assessed by immunoblot using a PKC-specific phospho-substrate antibody. Washed human platelets were pre-incubated with 0.5 mM apocynin, then treated for further 5 min with 200 μ M dRP (or vehicle solution) before stimulation with 0.05 unit/ml thrombin or mock stimulation with vehicle solution for 5 min in the presence of 1 mM EGTA and stirring (700 rpm). Following platelet lysis, protein extracts were separated by SDS-PAGE and the membranes were immunostained. The immunoblot presented here is representative of four independent experiments.