

ORIGINAL ARTICLE

cGMP signaling inhibits platelet shape change through regulation of the RhoA-Rho Kinase-MLC phosphatase signaling pathway

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To cite this article: Aburima A, Walladbegi K, Wake JD, Naseem KM. cGMP signaling inhibits platelet shape change through regulation of the RhoA-Rho Kinase-MLC phosphatase signaling pathway. *J Thromb Haemost* 2017; **15**: 1668–78.

Essentials

- Platelet shape change requires cytoskeletal rearrangement via myosin-mediated actin contraction.
- We investigated whether nitric oxide (NO) affected thrombin-induced platelet shape change.
- NO inhibits shape change, RhoA/ROCK signalling and myosin light chain (MLC) phosphorylation.
- NO promotes MLC phosphatase activity, thus prevents MLC phosphorylation and shape change.

Summary. *Background:* Platelet shape change, spreading and thrombus stability require activation of the actin cytoskeleton contractile machinery. The mechanisms controlling actin assembly to prevent unwanted platelet activation are unclear. *Objectives:* We examined the effects of nitric oxide on the signaling pathways regulating platelet actin-myosin activation. *Results:* S-nitrosoglutathione (GSNO) inhibited thrombin-induced platelet shape change and myosin phosphorylation of the myosin light chain (MLC). Because thrombin stimulates phospho-MLC through the RhoA/ROCK dependent inhibition of MLC phosphatase (MLCP) we examined the effects of NO on this pathway. Thrombin caused the GTP loading and activation of RhoA, leading to the ROCK-mediated phosphorylation of MLCP on threonine 853 (thr⁸⁵³), which is known to inhibit phosphatase activity. Treatment of platelets with GSNO blocked ROCK-mediated increases in phosphoMLCP-thr⁸⁵³ induced by thrombin. This effect was mimicked by the direct activator of protein kinase G, 8-pCPT-PET-

cGMP, and blocked by the inhibition of guanylyl cyclase, but not inhibitors of protein kinase A. Further exploration of the mechanism demonstrated that GSNO stimulated the association of RhoA with protein kinase G (PKG) and the inhibitory phosphorylation (serine188) of RhoA in a cGMP-dependent manner. Consistent with these observations, *in vitro* experiments revealed that recombinant PKG caused direct phosphorylation of RhoA. The inhibition of RhoA by GSNO prevented ROCK-mediated phosphorylation and inhibition of MLCP activity. *Conclusions:* These data suggest novel crosstalk between the NO-cGMP-PKG and RhoA/ROCK signaling pathways to control platelet actin remodeling.

Keywords: cyclic GMP; myosin light chain phosphatase; nitric oxide; platelets; protein kinase A; RhoA GTP-binding protein.

Introduction

Platelets circulate in the blood stream, monitoring vessel wall integrity. At sites of vascular injury, exposure of platelets to the extra cellular matrix (ECM) proteins collagen and von Willebrand factor (VWF) leads to platelet adhesion activation and spreading. Platelet activation is a highly orchestrated process that requires the reorganization of the cell cytoskeleton and dramatic morphological changes that are underpinned by a coordinated response between signaling molecules and a network of actin-binding proteins [1]. Foremost amongst these is myosin IIa, which when phosphorylated on its light chains (MLCs) can interact directly with actin filaments to drive shape change. The phosphorylation status of MLCs is controlled through the opposing actions of MLC kinase (MLCK) and MLC phosphatase (MLCP). Intracellular Ca²⁺ mobilization upon platelet activation leads to MLC phosphorylation at serine 19 (phosphoMLC-ser¹⁹) via MLCK. This phosphorylation intensifies an actomyosin contractile response that drives platelet adhesion and

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Received: 30 November 2016

Manuscript handled by: J. Heemskerk

Final decision: P. H. Reitsma, 11 April 2017

secretion [2,3]. Platelet activation through $G_{\alpha 12/13}$ protein coupled receptors (GPCRs) triggers activation of the small GTPase, RhoA, through membrane localization and GTP binding [4].

Membrane-bound RhoA associates with, and activates, Rho-associated, coiled-coil containing protein kinase (ROCK). A primary target of ROCK signaling is MLCP, with which it forms a complex to inhibit phosphatase activity. The outcome of this inhibition is to dynamically potentiate MLC ser¹⁹ phosphorylation because of the unopposed activity of MLCK. Platelet MLCP is composed of a 38-kD protein phosphatase 1c δ (PP1c δ) catalytic subunit, a 130-kD myosin-phosphatase targeting subunit 1 (MYPT1) and a 20-kD regulatory subunit of unknown function. MYPT1 acts as a molecular scaffold that localizes the activity of PP1c δ to myosin [5]. MYPT1 is targeted by multiple kinases, including ROCK, PKA, PKG and ILK [6–8]. These kinases potentially target multiple phosphorylation sites in MYPT-1, indicative of multiple layers of regulation. The best characterized of these kinases is ROCK, which induces MYPT1 phosphorylation on threonine (thr)⁸⁵³ and thr⁶⁹⁶ associated with inhibition of MLCP activity [9]. Previously, we have shown that platelet stimulation with thrombin increases MYPT1 thr⁸⁵³ phosphorylation, causes PP1c δ disassociation from MYPT1 and inhibits its activity [10]. However, the mechanisms that modulate the inhibitory phosphorylation of MLCP and help maintain platelets in their quiescent state remain poorly understood.

Platelet activation is controlled by cyclic nucleotide signaling pathways downstream of endothelial-derived nitric oxide (NO) and prostacyclin (PGI₂). Cyclic cGMP and cyclic AMP-dependent kinases have overlapping target specificity, but also synergise, indicating diversity in substrate selection [11]. Previously, we have found that cAMP signaling targets platelet shape change through regulation of the RhoA/ROCK/MLCP [10]. We have also demonstrated that NO prevents platelet cytoskeletal rearrangement in spreading platelets induced by collagen and VWF that was associated with diminished phosphorylation of MLC [12,13], although the mechanism remained unclear. In the present study we explored the possibility that cGMP signaling regulated platelets through a mechanism analogous to cAMP signaling by targeting MLCP activity. Our data demonstrate that NO prevents RhoA activation and downstream inhibitory phosphorylation of MYPT1 to modulate platelet shape change.

Methods

Reagents

The following antibodies were used: anti-phospho-VASPs²³⁹, anti-MYPT1, anti-phospho-MYPT1-thr⁸⁵³ (Cell Signalling Technology, Hitchin, UK); anti-PKG antibody

(BD Transduction Laboratories, Lexington, KY, USA); anti- β -tubulin (Upstate Biotechnology, Dundee, UK); and anti-phospho-RhoA-ser¹⁸⁸ (Santa Cruz, Calne, UK). Y27632, 1,2-bis-(*o*-aminophenoxy) ethane-tetra-acetic acid tetra-(acetoxymethyl) ester (BAPTA-AM) was from Calbiochem (Nottingham, UK). 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-CPT-cAMPS) and 8-(4-Chlorophenylthio)- β -phenyl-1, N²-ethenoguanosine-3', 5'-cyclic monophosphate (8-pCPT-PET-cGMP) were from Biolog (Bremen, Germany). RhoA pull-down kit was from Cytoskeleton (Peterborough, UK). Para-nitrophenyl phosphate was from New England Biolabs (Hitchin, UK). All other reagents were obtained from Sigma Ltd (Poole, UK).

Platelet isolation

Human blood was taken from drug-free volunteers into acid citrate dextrose (29.9 mM Na₃C₆H₅O₇, 113.8 mM glucose, 72.6 mM NaCl and 2.9 mM citric acid, pH 6.4). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200 \times g at 20 °C for 20 min. PRP was treated with citric acid (0.3 mM) and indomethacin (20 μ M) and centrifuged at 800 \times g for 12 min. The platelet pellet was then suspended in wash buffer (36 mM citric acid, 10 mM EDTA, 5 mM glucose, 5 mM KCl, 9 mM NaCl) and centrifuged at 800 \times g for 12 min. Platelets were resuspended (3 \times 10⁸ platelets mL⁻¹) in modified Tyrode's buffer (150 mM NaCl, 5 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.55 mM NaH₂PO₄, 7 mM NaHCO₃, 2.7 mM KCl, 0.5 mM MgCl₂, 5.6 mM glucose, pH 7.4) supplemented with apyrase (2U mL⁻¹), indomethacin (10 μ M) or EGTA (1 mM) unless otherwise stated.

Platelet shape change

Washed platelets (2.5 \times 10⁸ platelets mL⁻¹) were pre-incubated with EGTA (1 mM), apyrase (2 U mL⁻¹) and indomethacin (10 μ M). S-nitrosoglutathione (GSNO) (0.1–20 μ M) was added to platelets for 2 min prior to stimulation with thrombin (0.05 U mL⁻¹) and shape change recorded as previously described [10,12].

cAMP measurement

Washed platelets (2 \times 10⁸ platelets mL⁻¹) were treated with PGI₂ for 1 min or GSNO for 2 min at indicated concentrations and reactions were terminated by addition of ice-cold lysis buffer. cAMP levels were assayed with a commercially available enzyme immunoassay system following the manufacturer's instructions.

RhoA Pull-down assay

Washed platelets (5 \times 10⁸ platelets mL⁻¹) were treated with thrombin, in the presence and absence of GSNO

(10 μM), at 37 °C with stirring for 1 min before stopping the reaction with an equal volume of lysis buffer. Lysates (300 μg) were incubated for 90 min at 4 °C with Rhotekin-RBD-beads (25 μg). Bead pellets were washed once and Laemmli buffer was added prior to immunoblotting.

In vitro kinase assay

Recombinant human full-length active PKG was incubated with recombinant human His tagged-RhoA (55 ng) in kinase buffer (25 mM MOPS, pH7.2, 12.5 mM glycerol-2-phosphate, 25 mM MgCl_2 , 5 mM EGTA, 2 mM EDTA and 0.25 mM DTT) supplemented with ATP (400 μM) at 37 °C for 15 min. The reaction was stopped by addition of Laemmli buffer to the mixture for further immunoblot analysis.

Immunoprecipitation and immunoblotting

Washed platelets (8×10^8 platelets mL^{-1}) were treated with thrombin, in the presence or absence of GSNO, and incubated at 37 °C with stirring for the appropriate time. Reactions were stopped by addition of an equal volume of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Igepal, 1 mM phenylmethanesulfonyl fluoride, 2.5 mM Na_3VO_4 , phosphatase cocktail inhibitor, protease cocktail inhibitor, pH7.4). Lysates were incubated overnight at 4 °C with anti-PKG (1 μg) or IgG control antibodies, with constant rotation in the presence of protein A Sepharose beads. To prepare whole cell lysates platelets were then treated with the appropriate reagents before termination of the reactions with Laemmli buffer. Immunoprecipitates or lysates were separated by SDS-PAGE and analysed by immunoblotting as previously described [14]. The following primary antibodies were used: anti-phospho-VASP-ser²³⁹, anti-phospho-RhoA-ser¹⁸⁸ (both 1 : 1000), anti-RhoA and anti-phospho-MYPT1-thr⁸⁵³ (both 1 : 250).

Measurement of myosin phosphatase activity

Myosin phosphatase activity was determined as previously described [15]. PP1 δ was immunoprecipitated using anti-PP1 δ antibody from unstimulated platelets or platelets stimulated with thrombin in the presence or absence of GSNO. Immunoprecipitated PP1 δ was incubated for 1 h at room temperature (RT) with 5 mM pNPP in 100 μL reaction buffer (4 mM Tris-base, 2 mM KCl, 3 mM MgCl_2 , 0.1 mM MnCl_2 , 0.1 mg mL^{-1} BSA, 2 mM DTT, pH8.1). The reaction was stopped with 5 mM NaOH and absorbance was measured at 405 nm. Specific PP1 δ activity was obtained by subtracting the activity in the non-immune IgG IP from the activity in the PP1 δ IP. To verify that each sample had equal amounts of PP1 δ , samples were collected post-assay and analysed by immunoblotting for total PP1 δ .

Measurement of intracellular calcium flux

Washed platelets (1×10^8 platelets mL^{-1}) were loaded with the fluorescent probe Fura-3,AM at 37 °C for 30 min in the dark. Subsequently, platelets were washed with modified Tyrode's buffer and resuspended at 1×10^6 platelets mL^{-1} . Two mL of platelets were transferred into a quartz cuvette, which was placed in a luminescence spectrometer (Photon Technology International, Edison, NJ, USA). Calcium flux was measured by recording the ratio of fluorescence emitted at 530 nm after sequential excitation at 340 and 380 nm and expressed as fluorescence intensity (counts per second).

Statistical analysis

Results are expressed as means \pm SEM and statistical analyses were undertaken using Prism 6.0 (GraphPad, La Jolla, CA, USA). Differences between samples were determined using unpaired Student's *t*-test. The results were considered significant when *P* values were < 0.05 .

Results

S-nitrosoglutathione inhibits shape change and phosphorylation of MLC induced by thrombin

Platelet shape change is the earliest functional response following activation and is associated with cytoskeletal rearrangement. Under conditions that abrogated the effects of secondary signaling through ADP, TxA_2 and integrins, thrombin (0.05 U mL^{-1}) induced platelet shape change (Fig. 1A), which was associated with increased phosphorylation of MLC at serine19 (phospho-MLC-ser¹⁹) (Fig. 1B). When platelets were treated with GSNO (0.01–10 μM) prior to stimulation with thrombin, shape change was modulated and abolished at 10 μM (Fig. 1A). GSNO also caused a significant concentration-dependent inhibition of MLC phosphorylation (Fig. 1B). The inhibition of phosphorylation was evident at 2 min and was maintained for up to 60 min (Fig. 1C). To verify that the effect of NO on shape change was not specific to thrombin only, we also performed similar experiments in the absence of apyrase and indomethacin. Thrombin induced platelet shape change that was associated with an increase in MLC phosphorylation (Figure S1Ai). Pretreatment of platelets with GSNO caused an inhibition of platelet shape change and MLC phosphorylation (Figure S1Aii). Hence, NO inhibits shape change and MLC phosphorylation under conditions where all three agonists known to drive these responses are present [16,17].

Our previous work and that of others has shown that these concentrations of GSNO cause a rapid increase in cGMP [13]. We next examined the role of both cAMP and cGMP signaling downstream of GSNO treatment. In

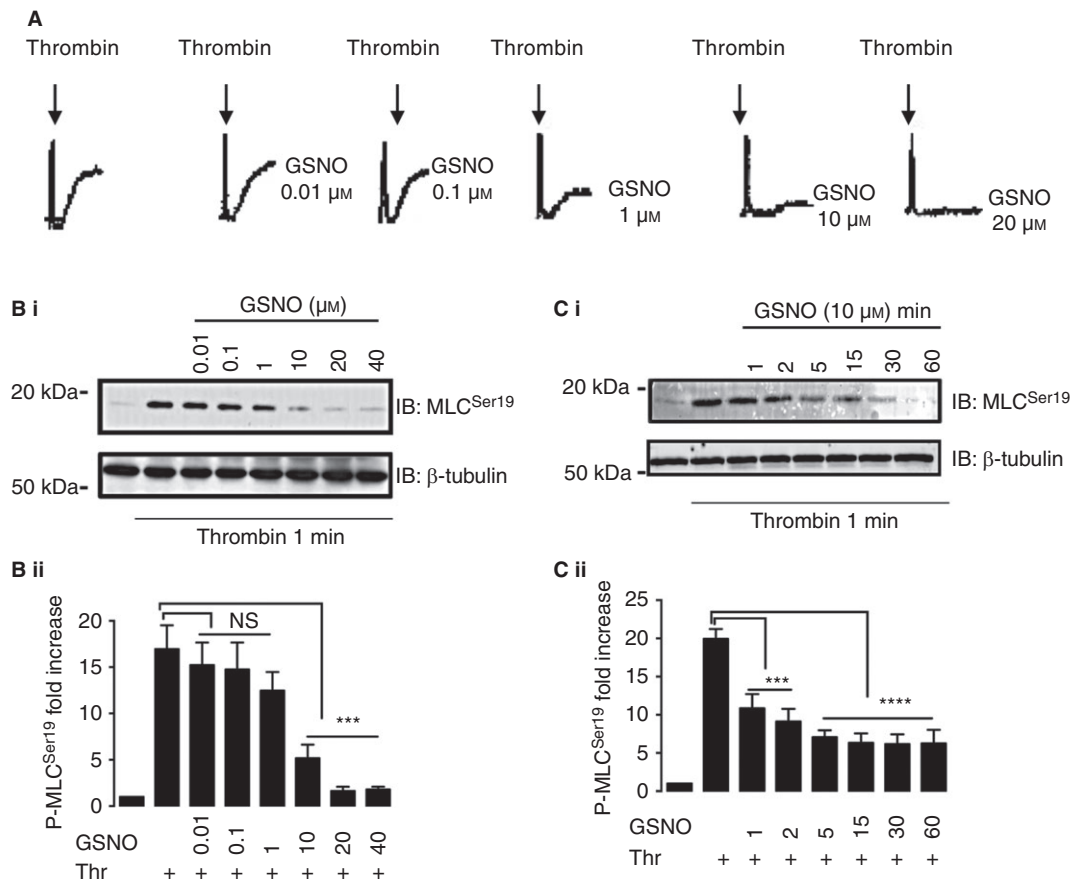


Fig. 1. Nitric oxide (NO) regulates thrombin-induced platelet shape change and myosin light chain (MLC) phosphorylation. (A) Washed platelets (3×10^8 platelets per milliliter) were preincubated with apyrase (2 U mL^{-1}), indomethacin ($10 \mu\text{M}$) and EGTA (1 mM), followed by stimulation with thrombin (0.05 U mL^{-1}) in the presence and absence of GSNO (0.01 – $20 \mu\text{M}$) and traces recorded for 1 min. (B) Washed platelets (3×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence or absence of GSNO (0.01 – $40 \mu\text{M}$) and phospho-MLC^{Ser19} was assessed by immunoblotting. (C) Washed platelets (3×10^8 platelets per milliliter) were treated with GSNO $10 \mu\text{M}$ for up to 60 min prior to stimulation with thrombin (0.05 U mL^{-1}) for 1 min and phospho-MLC^{Ser19} was assessed by immunoblotting.

the first instance, blocking sGC with ODQ [18] abolished the ability of GSNO to inhibit phosphorylation of MLC (Fig. 2A). By contrast, the effect of GSNO on (phospho-MLC-ser¹⁹) was maintained in the presence of PKA inhibitor Rp-8-CPT-cAMPS ($250 \mu\text{M}$) [19] (Fig. 2A). Similar results were obtained with KT-5720 ($20 \mu\text{M}$), a structurally diverse PKA inhibitor (not shown). These inhibitors blocked the phosphorylation of vasodilator-activated phosphoprotein (VASP) serine157 induced by PGI₂, confirming their specificity in platelets (Figure S1). In the second instance we used 8-pCPT-PET-cGMP ($5 \mu\text{M}$) as a cell-permeable activator of PKG that shows little cross-reactivity with cAMP signaling [20]. The cGMP analogue inhibited thrombin-induced MLC phosphorylation, which was unaffected by both ODQ and inhibitors of PKA (Fig. 2B). Importantly, ODQ also prevented GSNO from inhibiting thrombin-induced shape change (Fig. 2C). Thus, GSNO ($10 \mu\text{M}$) abolished thrombin-induced shape change and MLC phosphorylation through a mechanism that involves cGMP signaling.

GSNO targets both Ca²⁺-dependent and RhoA/Rho kinase pathways activated by thrombin

Several studies, including our own, have shown that thrombin stimulates shape change and phosphorylation of MLC through both Ca²⁺-dependent and RhoA/ROCK (Ca²⁺-independent) dependent pathways [10,14,21]. We have previously shown that cAMP signaling targets both of these pathways independently of each other [10] and wished to determine whether the same was true for cGMP. To achieve this we used BAPTA-AM to chelate intracellular Ca²⁺ and the ROCK inhibitor Y27632 ($10 \mu\text{M}$) [22]. We hypothesized that using these inhibitors alone and in combination with GSNO would allow us to determine which pathways were targeted by cGMP signaling. Thrombin (0.05 U mL^{-1}) induced shape change was partially inhibited by BAPTA-AM ($20 \mu\text{M}$) alone and Y27632 ($10 \mu\text{M}$) alone, but ablated when they were used in combination. Next, we treated platelets with BAPTA-AM ($20 \mu\text{M}$) alone and stimulated with thrombin; we

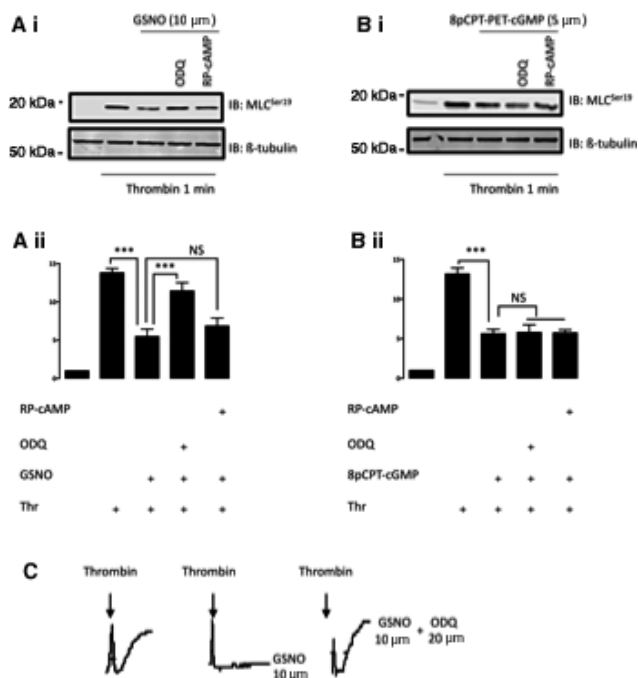


Fig. 2. Nitric oxide (NO) inhibits myosin light chain (MLC) phosphorylation in a cGMP-dependent manner and not cAMP-dependent manner. Washed platelets (3×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence or absence of GSNO ($10 \mu\text{M}$) (A) or 8-pCPT-PET-cGMP ($5 \mu\text{M}$) (B), ODQ ($20 \mu\text{M}$) and Rp-8-CPT-cAMPS ($250 \mu\text{M}$) and phospho-MLC^{ser19} was assessed by immunoblotting. (i) Representative immunoblots from four independent experiments. (ii) Densitometry of MLC^{ser19} phosphorylation from four different experiments. (C) Washed platelets (3×10^8 platelets per milliliter) were preincubated with apyrase (2 U mL^{-1}), indomethacin ($10 \mu\text{M}$) and EGTA (1 mM), followed by stimulation with thrombin (0.05 U mL^{-1}) in the presence and absence of GSNO ($10 \mu\text{M}$) and ODQ ($20 \mu\text{M}$).

reasoned that under these conditions only the ROCK pathway would be active, allowing examination of the effects of cGMP on ROCK signaling in isolation. BAPTA-AM alone reduced shape change, but this was abolished in combination with GSNO (Fig. 3A). Inhibition of the ROCK pathway with Y27632 ($10 \mu\text{M}$) was then used to study the effects of cGMP on the isolated Ca^{2+} -dependent pathway. Under these conditions shape change was again reduced but not blocked (Fig. 3A), whereas the combination of Y27632 and GSNO also abolished shape change (Fig. 3A).

To confirm that the effects on shape change occurred through the regulation of myosin IIa we next examined the phosphorylation of MLC. Thrombin-induced phosphoMLC^{ser19} was partially, but significantly, inhibited by both BAPTA-AM ($20 \mu\text{M}$) alone and Y27632 ($10 \mu\text{M}$) alone. The phosphorylation was abolished when the inhibitors were used in combination. Consistent with the shape change experiments we found that when GSNO ($10 \mu\text{M}$) was used in combination with either of the inhibitors the phosphorylation was ablated (Fig. 3B). These data suggest that cGMP signaling targets both the RhoA/ROCK and

Ca^{2+} -dependent pathways independently of each other to modulate platelet function. To determine whether GSNO inhibits Ca^{2+} -dependent signaling, we measured Ca^{2+} flux in thrombin-stimulated platelets. Treatment of platelets with BAPTA-AM resulted in a concentration-dependent inhibition of thrombin-induced MLC phosphorylation (Figure S1C). The highest concentration used ($20 \mu\text{M}$) also led to intracellular Ca^{2+} chelation. Consistent with this, and in agreement with previous reports [23,24], treatment of platelets with GSNO significantly inhibited thrombin-induced Ca^{2+} flux (Figure S1D).

GSNO inhibits thrombin-induced activation of RhoA

In the next series of experiments we examined the effects of cGMP signaling on the RhoA/ROCK-dependent signaling pathway. We first investigated if cGMP signaling targeted RhoA directly using a GTP-RhoA pull-down assay [25]. Thrombin (0.05 U mL^{-1}) elevated the levels of GTP-bound RhoA (Fig. 4A), which was abolished if platelets were pretreated with GSNO ($10 \mu\text{M}$). To confirm that our observation was cGMP mediated, we show that the inhibition of RhoA activation by GSNO was prevented by the sGC inhibitor ODQ (Fig. 4A). In contrast, Y27632 and BAPTA-AM had no effect on RhoA activation (data not shown), indicating that cGMP signaling targets RhoA activation independently of any potential effects on ROCK activity and Ca^{2+} flux. The phosphorylation of RhoA on serine¹⁸⁸ negatively regulates RhoA activity either through the inhibition of the GTPase activation or prevention of its membrane compartmentalization. Because PKG can phosphorylate RhoA *in vitro* [26], we hypothesized that PKG-mediated phosphorylation of RhoA may account, at least in part, for the inhibition of RhoA by cGMP signaling. GSNO (0 – $10 \mu\text{M}$) induced a concentration-dependent increase in phospho-RhoA-ser¹⁸⁸, with maximal phosphorylation observed at $10 \mu\text{M}$ (Fig. 4B). Phosphorylation of RhoA in response to GSNO occurred within 60 s and was maintained for 60 min (longest time tested) (Fig. 4C). In comparison, the phosphorylation of VASP-ser²³⁹ returned to basal after 45 min, suggesting that the phosphorylation of RhoA that was maintained for significantly longer may be regulated in a distinct manner.

Previously we demonstrated that RhoA can be targeted by cAMP signaling. Because several studies have suggested that NO can signal through both cAMP and cGMP-dependent pathways [27], it was important to clarify which pathway was responsible. To dissect which pathway downstream of NO controlled RhoA activity we used a multi-layered approach. Firstly, we measured intracellular cAMP levels in platelets treated with either PGI₂ or GSNO. PGI₂ (50 nM) at a concentration we have shown to induce RhoA phosphorylation caused a significant increase in cAMP concentrations to $2117 \pm 363 \text{ fmol}/10^8$ platelets ($P = 0.004$ vs. basal). By contrast,

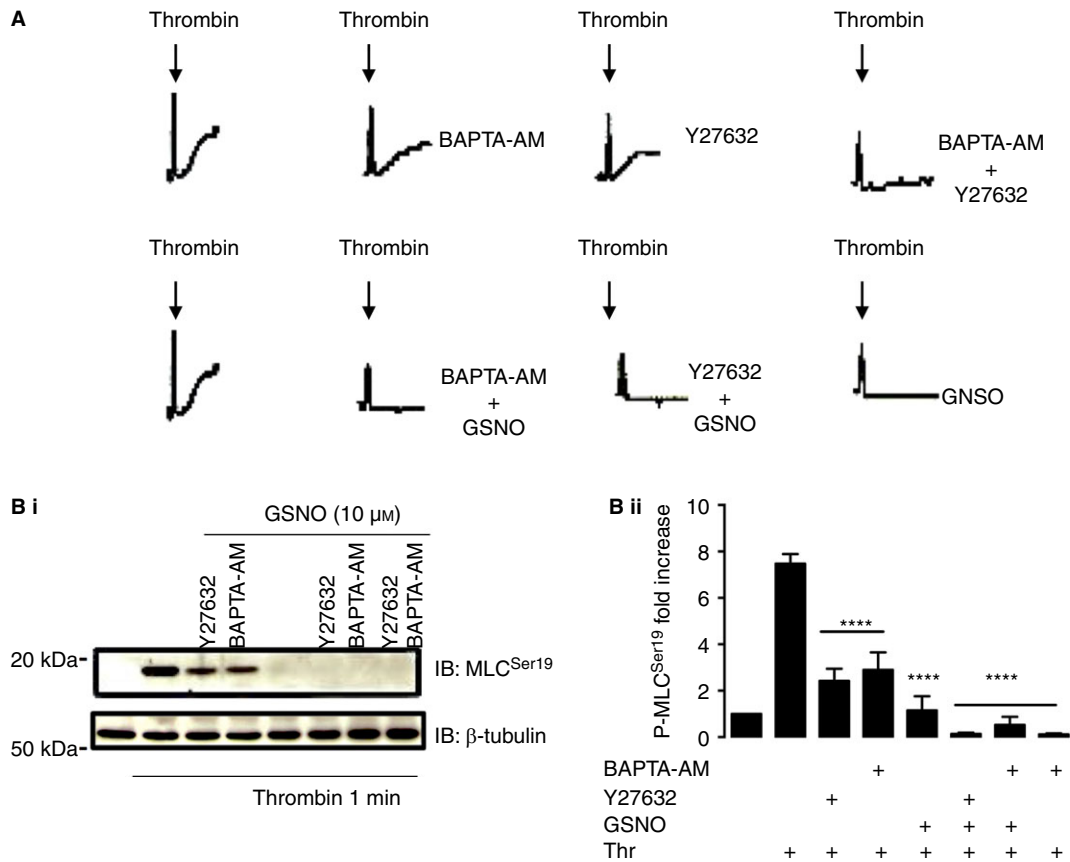


Fig. 3. Nitric oxide (NO) inhibits platelet shape change and myosin light chain (MLC) phosphorylation by targeting Ca^{2+} and RhoA/ROCK pathways. (A) Washed platelets (3×10^8 platelets per milliliter) were preincubated with apyrase (2 U mL^{-1}), indomethacin ($10 \mu\text{M}$) and EGTA (1 mM), followed by stimulation with thrombin (0.05 U mL^{-1}) in the presence and absence of GSNO ($10 \mu\text{M}$), Y27632 ($10 \mu\text{M}$) and BAPTA-AM ($20 \mu\text{M}$) and traces recorded for minute. (B) As in (A) except that phospho-MLC^{Ser19} was assessed by immunoblotting. [Color figure can be viewed at wileyonlinelibrary.com]

GSNO ($10 \mu\text{M}$) did not increase cAMP levels ($141 \pm 29 \text{ fmol}/10^8$ platelets, $P = 0.3$ vs. basal) above basal levels (Fig. 5A). Secondly, the sGC inhibitor ODQ ($20 \mu\text{M}$), but not the PKA inhibitors Rp-8-CPT-cAMPS ($250 \mu\text{M}$) and KT-5720 ($20 \mu\text{M}$) (not shown), prevented the phosphorylation of RhoA by GSNO ($10 \mu\text{M}$) (Fig. 5B). Thirdly GSNO-induced RhoA phosphorylation was reproduced by 8-pCPT-PET-cGMP ($5 \mu\text{M}$), which caused a significant phosphorylation of RhoA (Fig. 5C), which as expected was resistant to both sGC and PKA inhibitors. Together these data suggest that GSNO-induced RhoA phosphorylation is likely to be cAMP independent. In a fourth series of experiments we explored the potential role of PKG in the phosphorylation of RhoA. Immunoprecipitation of RhoA from platelets demonstrated that the PKG is associated with RhoA when platelets are treated with GSNO (Fig. 5D), suggesting that RhoA could be a direct target for PKG in platelets. Finally, to confirm that this association under physiological conditions could lead to the phosphorylation of RhoA by PKG, we performed an *in vitro* kinase assay. Incubation of recombinant human RhoA with recombinant active PKG resulted in the phosphorylation of RhoA on serine¹⁸⁸ (Fig. 5E).

GSNO modulates the inhibitory phosphorylation of MYPT1 by ROCK

ROCK is a downstream effector of RhoA that is proposed to drive platelet shape change and secretion through inhibition of MLCP [2]. Having established that cGMP signaling phosphorylated and inhibited RhoA, we examined how this influenced downstream signaling events. We first examined the effects of GSNO on MLCP activity through the immunoprecipitation of the PP1 δ subunit using a well-established approach [2]. Immunoprecipitation of PP1 δ demonstrated a basal activity of myosin phosphatase, which was reduced significantly when platelets were stimulated with thrombin (1 ± 0.0 to 0.5 ± 0.08 , $P = 0.006$ vs. basal). Pretreatment with GSNO prevented the inhibition of MLCP activity by thrombin, with activity remaining at basal levels (1.06 ± 0.03 , $P = 0.004$ vs. thrombin) (Fig. 6A). ROCK inhibits MLCP through the phosphorylation of MYPT1 on thr⁶⁹⁶ and thr⁸⁵³, the targeting subunit of MLCP, which inhibits the phosphatase [10]. We used the phosphorylation of the best-characterized site, thr⁸⁵³, as a marker of ROCK activity. The ability of GSNO to inhibit ROCK-mediated phosphorylation of MYPT1-

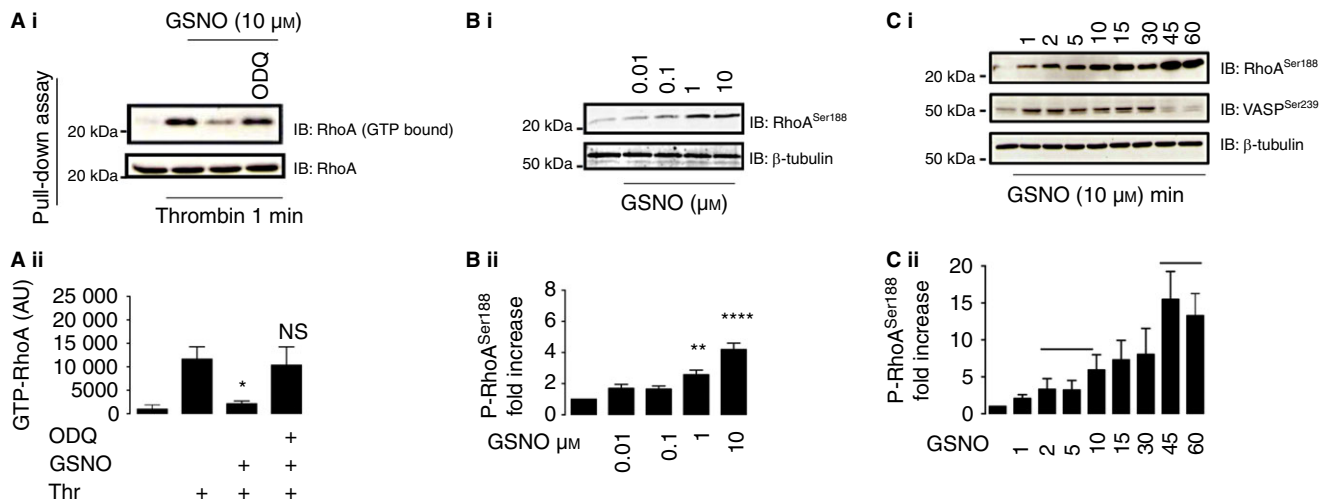


Fig. 4. Nitric oxide (NO) inhibits RhoA activation by inducing RhoA^{Ser188} phosphorylation. (A) Washed platelets (5×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence or absence of GSNO ($10 \mu\text{M}$) and ODQ ($20 \mu\text{M}$) and activated RhoA (GTP-RhoA) was assessed by using a pull-down assay with Rhotekin-RBD beads. Washed platelets (3×10^8 platelets per milliliter) were treated with GSNO (0.01 – $10 \mu\text{M}$) (B) and GSNO ($10 \mu\text{M}$) (C) for up to 60 min and phospho-RhoA^{Ser188} and phospho-VASP^{Ser239} were assessed by immunoblotting. (i) Representative immunoblots from four independent experiments. (ii) Densitometry analysis from four different experiments. [Color figure can be viewed at wileyonlinelibrary.com]

thr⁸⁵³ was evident at $1 \mu\text{M}$ but maximal at $10 \mu\text{M}$ (Fig. 6B). Using this same concentration of the NO donor we observed that the inhibition began within 30 s of exposure to GSNO and was maintained for 60 min (longest time tested) (Fig. 6C). Importantly, MYPT1-thr⁸⁵³ phosphorylation was abolished by Y27632, but not by BAPTA-AM (Fig. 6D), confirming the role of ROCK. The ability of GSNO to inhibit phosphorylation of MYPT1-thr⁸⁵³ was prevented by ODQ (Fig. 6E), consistent with the requirement for cGMP. To verify the role of PKG, experiments were repeated in the presence of the PKA inhibitor Rp-8-CPT-cAMPS, which has no effect on GSNO-mediated inhibition of MYPT phosphorylation.

Discussion

Platelet actin polymerisation is required for platelet shape change, secretion and stability of thrombi under flow. Understanding the mechanisms that control the polymerisation is critical to understanding how effective hemostasis is maintained. The small GTPase RhoA is crucial to these events, as exemplified by the platelet deficient in RhoA, which show altered shape change and function [28]. Previously we have shown that PGE₁ inhibits platelet shape change by targeting the RhoA/ROCK pathway in a PKA-dependent manner [10]. In the present study we expand these initial observations to demonstrate that cGMP signaling also targets RhoA to prevent inhibition of MLCP (disinhibition) and control MLC phosphorylation, suggesting that RhoA is a critical node for cyclic nucleotide-mediated inhibition of platelets.

We found that treatment of platelets with GSNO caused the ablation of shape change under stirring conditions,

consistent with previous observations showing that NO prevents platelet spreading [29]. This was mirrored by the inhibition of MLC phosphorylation, a key event in actin polymerisation. Shape change and the associated phosphorylation of MLC are driven through both Ca²⁺-dependent and independent pathways, and although we and others have established the effects of NO on Ca²⁺ signaling, our knowledge of inhibitory mechanisms by which NO inhibits platelets independently of effects on Ca²⁺ is unclear. Using BAPTA-AM to isolate RhoA/ROCK signaling we found that NO abolished both shape change and MLC phosphorylation driven by RhoA signaling. NO inhibited thrombin-induced intracellular Ca²⁺ flux to levels similar to the chelator BAPTA-AM. We could confirm that NO targeted RhoA directly because RhoA pull-down assays demonstrated that NO blocked the activation of RhoA upstream of ROCK. NO inhibits platelet function in a cGMP-dependent manner, evident by the lack of inhibition in GCKO platelets [30]. However, cGMP-independent mechanisms have also been documented [29], including through cAMP-dependent signaling and S-nitrosylation [31].

Of particular interest was the potential role of cAMP, which we have also shown to target RhoA signaling [10]. NO-induced cGMP has been proposed to inhibit PDE3A, leading to accumulation of cAMP and activation of PKA [32]. Indeed, one study has shown that NO-induced phosphorylation of VASP, a substrate for both PKA and PKG, was entirely dependent on PKA signaling [33]. Given this crosstalk between cyclic nucleotide signaling, it was possible that NO could inhibit RhoA activation through cAMP/PKA signaling. To dissect the cGMP-dependent and independent effects of NO on platelet shape change, we used the established sGC inhibitor

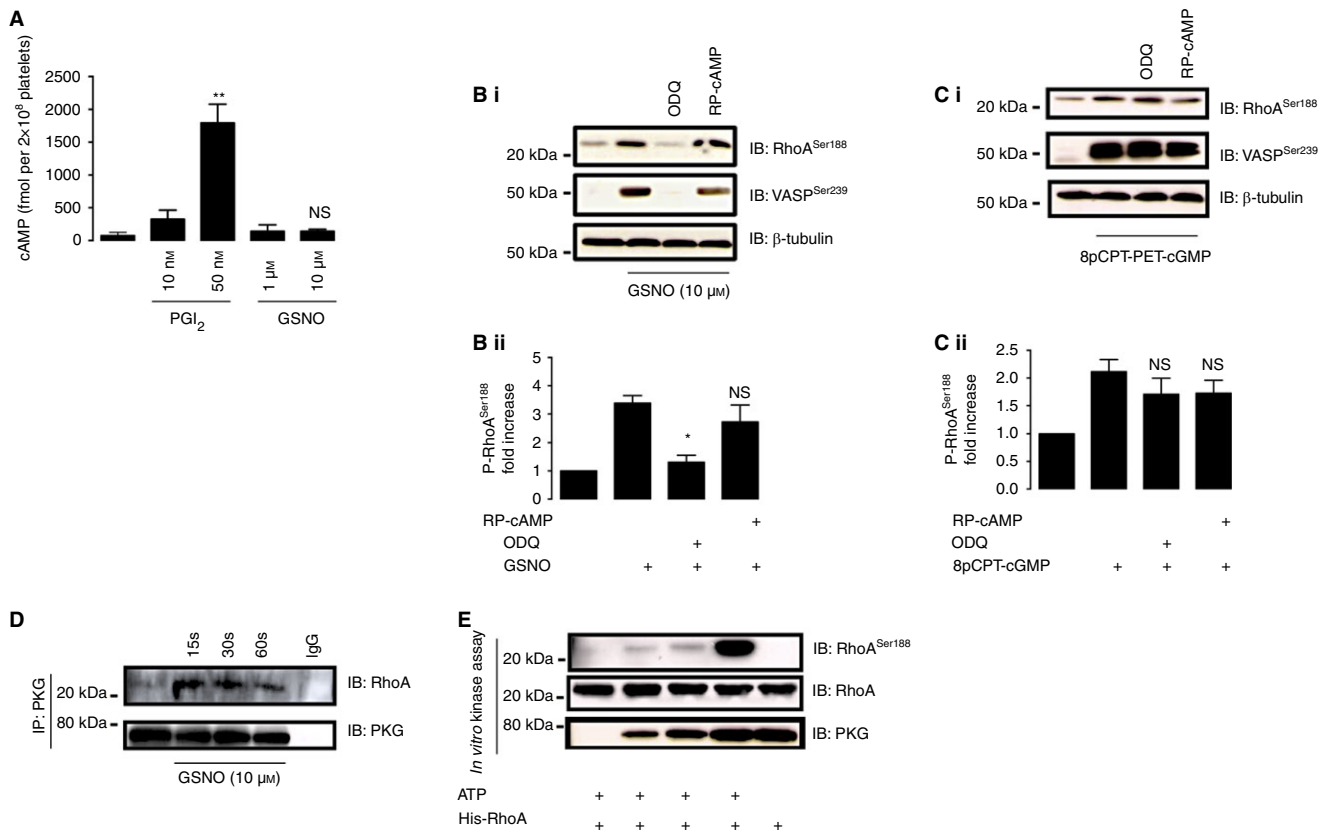


Fig. 5. Nitric oxide (NO) directly induces RhoA^{Ser188} phosphorylation in a cGMP-dependent, but not cAMP-dependent, manner. (A) Washed platelets (2×10^8 platelets per milliliter) were treated with PGI₂ (10–50 nM) or GSNO (1–20 μ M) and intracellular cAMP levels were measured. (B) Washed platelets (3×10^8 platelets per milliliter) were treated with GSNO (10 μ M) in the presence and absence of ODQ (20 μ M) and Rp-8-CPT-cAMPS (250 μ M) and phospho-RhoA^{Ser188} was assessed by immunoblotting. (C) As in (B) except washed platelets were treated with 8pCPT-PET-cGMP (5 μ M) instead of GSNO. (D) Washed platelets (8×10^8 platelets per milliliter) were treated with GSNO (10 μ M) for up to 1 min then lysed and RhoA was immunoprecipitated from the lysate overnight. RhoA and PKG β in the immunoprecipitates (IP) were detected by immunoblotting. (E) In lanes 2 to 5, recombinant active PKG β (1 to 5 ng) was incubated with recombinant His-tagged RhoA (55 ng) in the presence of adenosine triphosphate (ATP; 400 μ M) for 15 min at 37 °C. Reactions were stopped with 2 \times Laemmli buffer and RhoA^{Ser188} phosphorylation was assessed by immunoblotting. In lane 5, ATP was omitted from the reaction mixture. (i) Representative immunoblots from four independent experiments. (ii) Densitometry analysis from four different experiments. [Color figure can be viewed at wileyonlinelibrary.com]

ODQ [18]. Thrombin-induced platelet shape change, RhoA activation and MLC phosphorylation were inhibited by GSNO, which in turn was prevented by the presence of ODQ, suggesting a cGMP-dependent pathway. Given that cGMP could induce cAMP formation through inhibition of PDE3A, we measured cAMP concentrations in response to GSNO. Using a concentration of NO donor that completely inhibited RhoA activation, we found no significant increase in cAMP concentrations. Given newer models of cAMP signaling, it was also possible that a localized cAMP response [34–36], which would not be detected using cAMP measurement in platelet lysates, could have occurred, leading to PKA activation.

To account for this we examined the ability of GSNO to induce phosphorylation of RhoA and prevent the inhibitory phosphorylation of MYPT-1 in the presence of established PKA inhibitors. These inhibitors did not influence the actions of GSNO, suggesting strongly that cAMP/PKA signaling was not required for the actions of

GSNO. Having excluded a role for PKA signaling, we characterized the potential role of cGMP signaling. Given the lack of specific cell-permeable PKG inhibitors with reasonable potency [37], we confirmed the role of cGMP by using the membrane-permeable cGMP analog (8-pCPT-PET-cGMP). This agent served two purposes, both confirming a role for cGMP and acting as direct activator of PKG. Consistent with GSNO, 8-pCPT-PET-cGMP inhibited MLC phosphorylation and induced inhibitory phosphorylation of RhoA, actions that were unaffected by ODQ or PKA inhibitors. Although the pharmacological interventions were strongly suggestive of a key role for PKG, we went on to confirm the potential interactions of PKG and RhoA. The immunoprecipitation of RhoA revealed association of PKG with RhoA in response to GSNO. Although this does not prove that PKG phosphorylates RhoA, results from an *in vitro* PKG kinase assay showed that RhoA can be phosphorylated by PKG in the absence of other mediators.

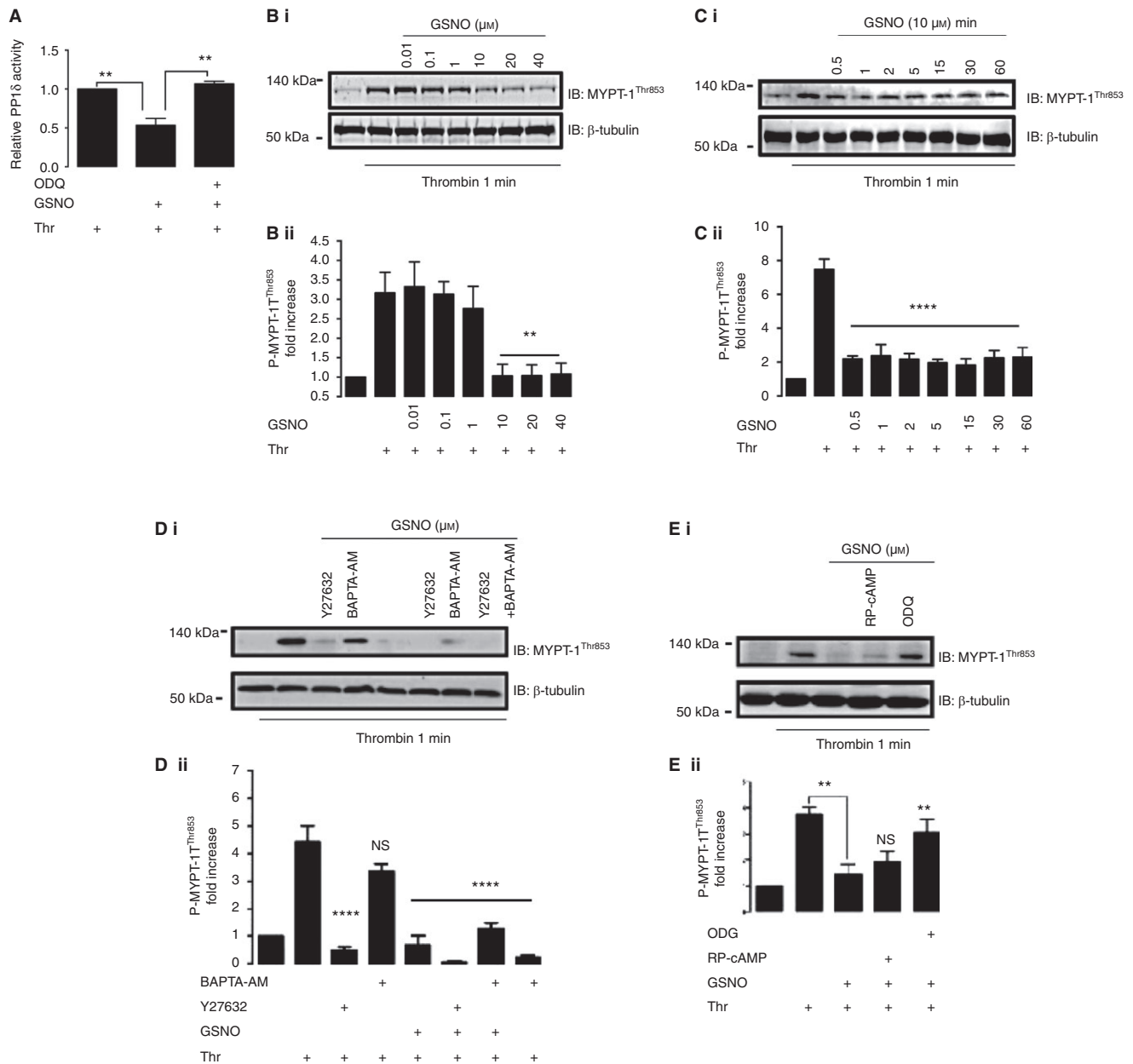


Fig. 6. Nitric oxide (NO) inhibits MYPT-1 phosphorylation by targeting the RhoA/ROCK pathway. (A) PP1 δ was immunoprecipitated and incubated for 1 h at room temperature with 5 mM para-nitrophenyl phosphate, and absorbance was measured at 405 nm. The graph is representative of four different experiments. (B) Washed platelets (5×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence and absence of GSNO (0.01–40 μM) and phospho-MYPT-1^{Thr853} was assessed by immunoblotting. (C) As in (B) except washed platelets were pretreated with GSNO (10 μM) for up to 60 min prior to stimulation with thrombin. (D) Washed platelets (5×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence and absence of GSNO (10 μM), Y27632 (10 μM) and BAPTA-AM (20 μM) and phospho-MYPT-1^{Thr853} was assessed by immunoblotting. (E) Washed platelets (5×10^8 platelets per milliliter) were stimulated with thrombin (0.05 U mL^{-1}) for 1 min in the presence and absence of GSNO (10 μM), ODQ (20 μM) and Rp-8-CPT-cAMPS (250 μM) and phospho-MYPT-1^{Thr853} was assessed by immunoblotting. (i) Representative immunoblots from four independent experiments. (ii) Densitometry analysis from four different experiments.

MLCP plays a key role in the regulation of actin polymerisation [38]. The phosphorylation of the MYPT-1 scaffolding component of this complex by multiple kinases controls its localization and activity through the formation of macromolecular complexes. Stimulation with thrombin causes ROCK-mediated inhibitory phosphorylation and disruption of the haloenzyme structure. Our current and

previous data suggest that cyclic nucleotide-dependent kinases, PKG and PKA, inhibit RhoA activation as a mechanism to prevent inhibition of MLCP, a process termed disinhibition. The fact that both kinases have the capacity to target RhoA signaling suggests that inhibition of this pathway is critical to their ability to modulate platelet function. What is still unclear is whether *in vivo* there is

a mechanism that allows PKG and PKA to differentially target RhoA, which could be either context or location dependent. Furthermore, it is known that PKA and PKG can phosphorylate MYPT-1 directly in smooth muscle cells [39,40], which may provide an additional and coordinated mechanism to control MLC phosphorylation; this requires further investigation. Given the emerging roles of AKAPs and the concept of spatiotemporal control of cyclic nucleotide signaling [35], it is also possible that PKA and PKG target individual pools of RhoA and ROCK. This issue of the compartmentalization of cyclic nucleotide signaling in platelets requires further exploration.

In conclusion, NO-induced cGMP signaling modulated RhoA/ROCK signaling in platelets, leading to the disinhibition of MLCP to control the phosphorylation of MLC. The identification of RhoA as a new target for cGMP signaling provides a novel mechanism of platelet inhibition by NO and adds to the increasing complexity of inhibitory signaling in blood platelets.

Addendum

A. Aburima performed experiments and analyzed and interpreted data. K. Walladbegi performed experiments. J. D. Wake performed experiments. K. M. Naseem designed research, analyzed and interpreted data, and wrote the manuscript.

Acknowledgements

This study was funded by The British Heart Foundation (PG/10/90/28636).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rp-8-CPT-cAMPS inhibits PGI₂-induced VASP phosphorylation.

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