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Inhibition of ADP-induced platelet adhesion to immobilised fibrinogen by nitric oxide: Evidence for cGMP-independent mechanisms

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ABSTRACT

Nitric oxide (NO) is an established regulator of platelet function, although the processes by which NO modulates platelet adhesion are unclear. We studied the importance of Ca^{2+} and phosphoinositol-3-kinase (PI3kinase) as targets for NO signalling, in the physiological context of platelet adhesion using adenosine diphosphate (ADP)-stimulated adhesion to immobilised fibrinogen. DPTA-NONOate induced a time and concentration-dependent inhibition of adhesion, and reduced protein tyrosine phosphorylation. The action of NO was cGMP-independent despite activation of the cGMP-signalling cascade, as evidenced by VASP phosphorylation. Furthermore, the cGMP-independent mechanism did not involve PKA. Platelet activation by ADP requires Ca^{2+} and PI3kinase-dependent signalling pathways. We examined the effect of NO on these pathways using two approaches. Firstly, we dissected the signalling pathways using the $P2Y_1$-receptor antagonist A3P5P, and secondly, directly inhibited Ca^{2+} mobilisation and PI3kinase activity. ADP-induced adhesion was reduced but not abolished by A3P5P, suggesting signalling from $P2Y_{12}$ can induce adhesion. NO further reduced adhesion in the presence of A3P5P, indicating that NO inhibited adhesion independently of any effects on Ca^{2+} mobilisation. Dimethyl bis-(o-aminophenoxy) ethane-tetraacetic acid (BAPTA) and wortmannin both partially inhibited ADP-induced adhesion, but completely abolished adhesion when used in combination, demonstrating that ADP-induced adhesion requires Ca^{2+} and PI3kinase-regulated pathways. Combination of either dimethyl-BAPTA or wortmannin with DPTA-NONOate enhanced inhibition of both the Ca^{2+} and PI3kinase-dependent pathways when compared to the levels of inhibition with either agent alone. Thus, we demonstrate that NO inhibits $\alpha_{IIb}\beta_3$-mediated adhesion, by targeting both Ca^{2+} and PI3kinase pathways in a cGMP-independent manner.

1. Introduction

The integrin $\alpha_{IIb}\beta_3$ facilitates platelet aggregation by binding soluble fibrinogen and mediates platelet adhesion to immobilised fibrinogen. $\alpha_{IIb}\beta_3$ is normally found on the platelet surface in a cryptic low affinity form unable to engage with its extracellular ligands. However, upon inside-out signalling induced by soluble platelet agonists such as adenosine

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diphosphate (ADP) or thrombin, it is converted to its high affinity form capable of ligand binding. In addition to being targeted by intracellular signalling cascades, $\alpha_{IIb}\beta_3$ can also initiate outside-in signalling. Signalling through $\alpha_{IIb}\beta_3$ results in activation of protein tyrosine phosphorylation signalling events and determines the extent of platelet spreading on vWF or fibrinogen surfaces and their resistance to detachment [1–3].

Platelet activation occurs through the stimulation of a large number of exquisitely integrated activatory signalling pathways that ensure rapid activation at the site of injury. However, platelets also require equally potent inhibitory signalling cascades to prevent excessive activation and to return them to their quiescent state after transient activation. Nitric oxide (NO) acts to both inhibit platelet adhesion to the ECM and limit platelet aggregation [4,5]. The mechanisms by which NO regulates platelet function are still not completely understood. NO diffuses into platelets activating soluble guanylyl cyclase, resulting in elevated levels of cGMP [6] and possibly cAMP [7]. cGMP-regulated signalling molecules inhibit a number of platelet activatory signalling pathways. However, cGMP-independent mechanisms regulating platelet function have also been demonstrated [8–10]. In suspended platelets, NO targets several proteins leading to the inhibition of platelet activation including inositol trisphosphate (IP_3) receptors [11], vasodilator–stimulated phosphoprotein (VASP) [12], Rap-Ib [13] and the thromboxane A_2 (TxA_2) receptor [14]. NO has been strongly implicated in the regulation of intracellular Ca^{2+} levels [8,15–20]. In suspended platelets, NO reduces Ca^{2+} release, which subsequently inhibits secretion and $\alpha_{IIb}\beta_3$ activation [18]. However, NO also inhibits RapIb phosphorylation [13] and phosphoinositol-3-kinase (PI3kinase) activity independently of changes in Ca^{2+} [21], indicating that NO targets both Ca^{2+} dependent and independent pathways regulating platelet activation.

While NO inhibits $\alpha_{IIb}\beta_3$ activation and fibrinogen binding in suspended platelets, the effect on the adhesion of platelets to immobilised fibrinogen is less clear. Indeed, it is yet to be established if NO targets common activatory signalling cascades in both suspended and adherent platelets. Here, we show that NO inhibits the adhesion of ADP-stimulated platelets to immobilised fibrinogen by a mechanism that is independent of cGMP. ADP-induced adhesion requires both Ca^{2+} and PI3kinase regulated pathways, both of which are targeted by NO. Thus, our data demonstrate that in the physiological context of platelet adhesion, NO targets at least two independent activatory pathways.

2. Materials and methods

2.1. Materials

The anti-phosphotyrosine monoclonal antibody (MoAb), 4G10 was purchased from Upstate Biotechnology Inc. (Milton Keynes, UK). The anti-Syk mouse MoAb was obtained from Santa Cruz (Herts, UK). Phospho-Ser^{239} VASP antibody was purchased from Cell Signalling Technology (Hitchin, UK). Anti-mouse and anti-rabbit IgG HRP were obtained from Amersham Biosciences (Bucks, UK). DC protein assay kit was purchased from Bio-Rad (Hemel-Hempstead, Herts, UK). Wortmannin, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 1,2-bis-(o-aminophenoxy)ethane-tetraacetic acid tetra-(acetoxymethyl) ester (BAPTA/AM) were obtained from Calbiochem (Nottingham, UK). All other reagents were from Sigma Ltd. (Poole, UK).

2.2. Isolation of human platelets

Human blood was taken from drug-free volunteers by clean venepuncture, via a 21-gauge butterfly needle using acid citrate dextrose (ACD; 29.9 mM sodium citrate, 113.8 mM glucose, 72.6 mM sodium chloride and 2.9 mM citric acid, pH 6.4) as anticoagulant. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 200 × *g* at 20 °C for 20 min. Platelets were isolated from the PRP by centrifugation at 800 × *g* at 20 °C for 12 min in the presence of prostaglandin E_1 (PGE_1; 50 ng/ml). The platelet pellet was resuspended at a concentration of 1×10^8platelets/ml in Tyrodes buffer (137 mM NaCl, 20 mM HEPES, 3.3 mM NaH_2PO_4, 2.7 mM KCl, 1 mg/ml BSA and 5.6 mM glucose, pH 7.4) unless otherwise stated [22]. When required, platelets were also incubated with dimethyl BAPTA (20 μM) for 30 min to chelate intracellular Ca^{2+}, indomethacin (10 μM) to block TxA_2 production, ODQ (20 μM) to inhibit soluble guanylyl cyclase (sGC) or wortmannin (100 nM) to inhibit PI3kinase activity. For some experiments KT5720 (5 μM) and Rp-cAMPS (0.5 mM) were used in combination to inhibit PKA activity.

2.3. Platelet adhesion assay

The platelet adhesion assay was based on the method developed by Bellavite et al. [23]. The 96-well microtitre plates were coated using 1 mg/ml fibrinogen or heat-inactivated human serum (HS) for 24 h at 4 °C and subsequently blocked for 30 min at room temperature using 5% HS. WP suspensions were incubated with inhibitors or agonists for the required time prior to each experiment and then 50 μl suspension were added to each well and left to adhere at 37 °C for the indicated time. After removal of non-adherent platelets, adherent platelets were incubated with 0.1 M citrate buffer containing 5 mM *p*-nitrophenol phosphate and 0.1% Triton X-100 (31 mM citric acid, 5 mM sodium citrate dehydrate, 5 mM *p*-nitrophenyl phosphate, 0.1% (v/v) Triton X-100, pH 5.4) for 1 h at room temperature. The reaction product was then visualised using 2N NaOH and measured at 405 nm. In preliminary studies, 96-well plates were checked by phase contrast microscopy and under the assay conditions used no aggregation was observed (not shown). None of the platelet reagents used had any effect on platelet acid phosphatase activity. Percentage inhibition was calculated by absorbance values induced by ADP (10 μM) compared to the absorbance in the induced by ADP in the presence of NO or other inhibitors.

2.4. Microscopy

Platelets (2×10^7 ml^{-1}) were added to fibrinogen-coated coverslips and incubated for 30 min at 37 °C. In some experiments, platelets were incubated with DTPA-NONOate (10 μM) for 5 min prior to addition to coverslips. After incubation, coverslips were washed two times with PBS to remove non-adherent platelets.

Adherent platelets were fixed with 4% paraformaldehyde and permeablised with 0.1% Triton-X-100 in PBS. Platelets were stained for F-actin using tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin and visualised using fluorescent microscope (Nikon Eclipse 80i). For each experiment, the number of platelets from eight random fields of view were added the results were then calculated as mean number of adherent platelet ± S.E.M. per 0.1 mm^2.

2.5. Immunoblotting studies

Platelets (5×10^8 ml^{-1}) were allowed to adhere to fibrinogen (1 mg/ml)-coated wells (six-well plates) in the presence or absence of NO for up to 60 min. Non-adherent platelets were removed and adherent platelets lysed by the addition of Laemmli sample buffer (1×). Protein content of the lysates was determined, and after accounting for fibrinogen, 20 μg of protein was loaded for immunoblotting on 10–18% polyacrylamide gradient gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, membranes were blocked with 10% BSA dissolved in Tris-buffered-saline-Tween (0.1%) (TBS-T). Antibodies were diluted in TBS-T containing 2% BSA. For phosphotyrosine immunoblots, membranes were initially incubated with anti-phosphotyrosine 4G10 (1:1000), stripped and reprobed with anti-Syk MoAb (1:2000). For VASP immunoblots, membranes were incubated with anti-phosphoserine^{239} VASP (1:1000). Following incubation with either primary MoAb membranes were subject to four 15 min washes with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10,000) for anti-phosphotyrosine and anti-Syk or HRP-conjugated anti-rabbit (1:10,000) for anti-phosphoserine VASP. Membranes were visualised using enhanced chemiluminescence. Stripping was performed by washing the membrane in TBS-T containing 5% mercaptoethanol and 2% SDS at 80 °C for 20 min. Densitometric analyses were performed using Scion Image program (NIH), and expressed as a ratio of phosphotyrosine to total Syk.

2.6. Measurement of protein concentrations

Protein concentrations were determined in platelet lysates using the BioRad protein assay, which is based on the Lowry assay, using bovine serum albumin as standard.

2.7. Statistical analysis

Results are expressed as means ± S.E.M. and were analysed using the Student's *t*-test or ANOVA for unpaired data as appropriate. The results were considered significant when p values were <0.05.

This investigation conforms with the principles outlined in the Declaration of Helsinki.

3. Results

3.1. NO inhibits adhesion to immobilised fibrinogen

In vivo circulating platelets are continually bathed in endothelial derived-NO. Hence, platelets were exposed to DPTA-NONOate, a donor with a half-life of 3 h, to ensure bioavailable NO was present throughout the experiment (confirmed using phosphoVASP; see Fig. 3). NO (0–100 μM)-induced a concentration-dependent inhibition of adhesion compared to ADP (10 μM) alone (Fig. 1A). DPTA-NONOate (0.1 μM) inhibited adhesion by 16 ± 4% ($p < 0.05$) while maximal inhibition (50 ± 6%) was induced by 10 μM ($p < 0.001$). Adhesion under these conditions was $\alpha_{IIb}\beta_3$-dependent, since adhesion was ablated by the presence of RGDS peptide (data not shown). Decomposed DPTA-NONOate (10 μM) did not inhibit adhesion, confirming that NO and not DPTA or decomposition products were responsible for the inhibitory actions. To exclude the possibility that NO inhibits platelet acid phosphatase activity rather than adhesion, we tested the effect of NO on acid phosphatase activity. We have

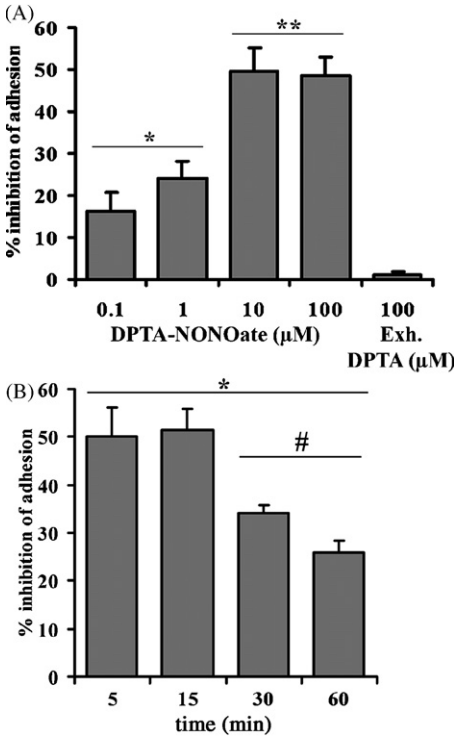

Fig. 1 – NO-mediated inhibition of ADP-induced adhesion to immobilised fibrinogen. (A) Washed platelets (1×10^8 platelets/ml) were preincubated for 10 min with DPTA-NONOate and allowed to adhere to fibrinogen-coated wells containing ADP (10 μM) for 15 min. Alternatively exhausted (exh.) DPTA-NONOate was used ($^{*}p < 0.05$, $^{**}p < 0.001$ compared to ADP alone). (B) Washed platelets were treated with DPTA-NONOate (10 μM) and allowed to adhere to fibrinogen-coated wells for up to 60 min. Platelet adhesion was determined by platelet acid phosphatase activity remaining in the well after removal of unbound platelets (see Section 2). The data are expressed as percentage inhibition of adhesion as compared to adhesion at each time point in the absence of DPTA-NONOate ($^{*}p < 0.05$ when compared to control value at each time point; $^{\#}p < 0.05$ when compared to 5 min inhibition value). Results are presented as mean ± S.E.M. of four independent experiments each performed in triplicate.

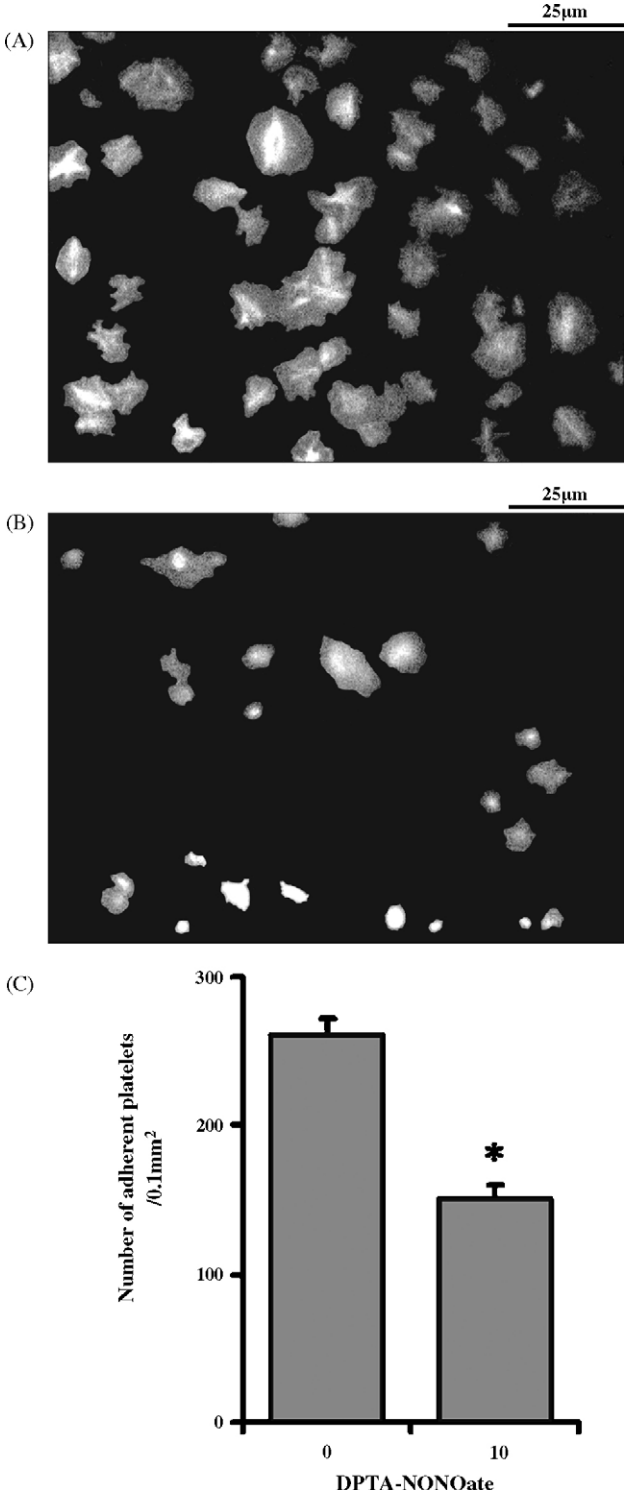

Fig. 2 – Visualisation of platelet adhesion to fibrinogen and inhibitory effect of NO. Platelets were mixed with ADP and placed on fibrinogen-coated coverslips for 15 min at 37 °C in absence (A) and presence (B) of DPTA-NONOate (10 μM). At the end of the incubation period, non-adherent platelets were removed by washing with PBS. The remaining adherent platelets were fixed, permeablised with 0.1% Triton, and stained for F-action with TRITC-conjugated phalloidin. The results are presented as mean number of adherent platelet ± S.E.M. per 0.1 mm^2 of four independent experiments (C). For each experiment, the number of platelets from eight random fields of view were combined ($^*p < 0.01$ compared to ADP alone).

found that NO at the concentrations used here does not alter acid phosphatase activity (data not shown), as recently demonstrated by Marcondes et al. [24]. Next, we sought to determine the time course of the inhibitory actions of NO. After 5 min, DTPA-NONOate (10 μM) inhibited platelet adhesion by 50 ± 6%, which was maintained for up to 15 min. However, at later time points inhibition progressively decreased significantly, such that at 60 min inhibition had fallen to 26 ± 3% ($p < 0.05$, compared to inhibition at 5 min), demonstrating sustained but reversible inhibition (Fig. 1B). Similar results were obtained with the structurally distinct NO-donor S-nitrosoglutathione (data not shown). The adhesion of platelets to fibrinogen was further investigated by staining of the actin cytoskeleton using TRITC-labelled phalloidin. Platelets adhered and spread on immobilised fibrinogen leading to the formation of actin stress fibres, but did not aggregate. The number of platelets forming stable adhesions on fibrinogen was 260 ± 12 per 0.1 mm^2, which was reduced significantly to 150 ± 10 per 0.1 mm^2 ($p < 0.01$) in the presence of DTPA-NONOate. Furthermore, the images suggest that NO also reduces spreading. Thus, consistent with our biochemical assessment of platelet adhesion, DTPA-NONOate (10 μM) inhibited platelet adhesion by 43 ± 5% (Fig. 2).

3.2. *NO induced inhibition of platelet adhesion is independent of sGC and PKA activity*

NO inhibits platelet aggregation through cGMP-dependent and independent mechanisms [8,9], however, it is unknown if the same is true for adhesion. To address this issue, we assessed the effects of the established sGC inhibitor ODQ [25] on DPTA-NONOate mediated inhibition of adhesion. In preliminary experiments, ODQ completely inhibited cGMP formation in response to NO as observed previously (not shown). DPTA-NONOate (10 μM) induced a 56 ± 5% inhibition of adhesion, but surprisingly the inclusion of ODQ (20 μM) did not reduce the anti-adhesive effects of DPTA-NONOate (Fig. 3A). Similar results were observed at the 60 min time point. To confirm that under our experimental conditions NO activated the cGMP/PKG signalling pathway, and that ODQ inhibited this process, we performed immunoblotting experiments for VASP phosphorylation (serine^{239}) an established target of PKG [26]. Immunoblotting of fibrinogen-bound platelets demonstrated that DPTA-NONOate increased VASP phosphorylation with phosphorylation already maximal at 15 min and maintained at 60 min, indicating that NO was stimulating PKG activity (Fig. 3B). Importantly, DPTA-NONOate-induced VASP phosphorylation was reduced to basal levels by ODQ (Fig. 3B). Interestingly ODQ alone caused minor phosphorylation of VASP, the significance of which is unclear. Despite this, the inhibitor consistently blocked NO induced formation of phospho-VASP. Thus, NO-mediated inhibition of adhesion under these conditions occurs via a cGMP-independent mechanism despite activation of the major NO signalling cascade.

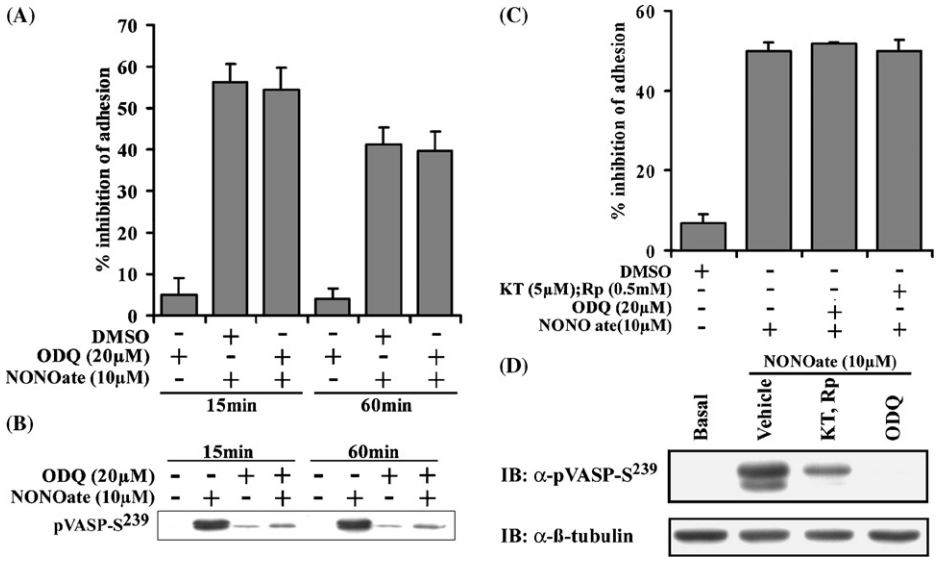

Fig. 3 – NO inhibits ADP-induced adhesion by a cGMP-independent mechanism. (A) Washed platelets (1×10^8 platelets/ml) were preincubated with ODQ (20 μM) or the PKA inhibitors KT5720 (5 μM) as indicated before preincubating for 10 min with DPTA-NONOate (10 μM) and allowed to adhere to fibrinogen-coated wells containing ADP (10 μM). The data are expressed as percentage inhibition of adhesion as compared to adhesion in the absence of DPTA-NONOate. Results are presented as mean ± S.E.M. of 10 independent experiments for ODQ and 3 independent experiments for KT5750/Rp, each performed in triplicate. (B) Washed platelets (5×10^8 platelets/ml) preincubated with DPTA-NONOate added to fibrinogen-coated wells (six-well plates) containing ADP (10 μM) in the presence and absence of ODQ (20 μM) and allowed to adhere for up to 60 min or for 15 min in the case of the PKA inhibitors. (I) Cells were lysed, separated by SDS-PAGE and immunoblotted for phosphoVASP-serine^{239}. The blot is representative of three independent experiments.

NO inhibits thrombin-induced shape change and VASP phosphorylation via PKA [7,27]. To investigate whether PKA activation account for the inhibitory effects of NO on platelet adhesion, platelets were preincubated with KT5720 and Rp-cAMPS (5 μM and 0.5 mM, respectively). PKA inhibition had no effect on DPTA-NONOate-induced inhibition of platelet adhesion (Fig. 3C). However, DPTA-NONOate-induced VASP phosphorylation was clearly reduced in the presence of KT5720 and Rp-cAMPS (Fig. 3D), confirming that NO signals at least in part via PKA.

3.3. *NO inhibits adhesion-induced tyrosine phosphorylation in platelets*

Platelet adhesion to fibrinogen induces $\alpha_{IIb}\beta_3$-mediated tyrosine phosphorylation and we sought to determine if NO could modulate these signalling processes. ADP-induced adhesion to immobilised fibrinogen after at 15 min increased phosphorylation of a number of bands with robust phosphorylation observed at apparent molecular weights of 72, 90 and 125 kDa (Fig. 4A). Beyond this time point adhesion induced tyrosine phosphorylation declined, illustrating the temporal nature of the signalling response. DTPA-NONOate (10 μM) reduced overall protein tyrosine phosphorylation (Fig. 4A). Densitometric analysis of individual protein bands demonstrated that DPTA-NONOate significantly reduced phosphorylation of the 72, 90 and 125 kDa bands after 15 min of adhesion ($p < 0.05$; Fig. 4B I–III). The effects of DPTA-NONOate on adhesion-induced phosphorylation at later time points were not significant because of the overall decline in tyrosine phosphorylation with time. Thus, in addition to inhibiting adhesion, NO modulates adhesion-induced tyrosine phosphorylation based signalling.

3.4. *The roles of Ca^{2+} mobilisation and PI3kinase in ADP-induced adhesion to fibrinogen: influence of NO*

On platelets ADP stimulates the G_q-coupled $P2Y_1$ receptor that is associated with Ca^{2+} mobilisation and shape change [28], and the Ca^{2+} independent G_i-coupled $P2Y_{12}$ receptor, which inhibits adenylyl cyclase and activates PI3kinase [29]. To examine whether NO differentially targeted these signalling cascades during platelet adhesion, we used two approaches, firstly, we dissected the Ca^{2+} and PI3kinase regulated pathways using P2Y receptor antagonists, and secondly, directly inhibited Ca^{2+} mobilisation and PI3kinase activity.

First, we established that $P2Y_{12}$ signalling was sufficient to induce adhesion to fibrinogen. In the presence of the established $P2Y_1$ inhibitor A3P5P (100 μM) [30,31], ADP-mediated adhesion was inhibited by 47 ± 3%. If platelets were also incubated with 2-ME-SAMP (1 mM) a $P2Y_{12}$ inhibitor adhesion was completely abolished. Likewise, preincubation of platelets with A3P5P and the PI3kinase inhibitor wortmannin (100 nM) ablated adhesion to fibrinogen (Fig. 5A). Hence, adhesion to fibrinogen was only partially dependent upon $P2Y_1$ receptor driven Ca^{2+} mobilisation, indicating that a significant proportion of the adhesion response required PI3kinase signalling from $P2Y_{12}$. Next, we incubated platelets with A3P5P (100 μM) in combination with DPTA-NONOate (10 μM). If NO were only targeting $P2Y_1$ receptor signalling, the level of inhibition of adhesion in the presence of the receptor

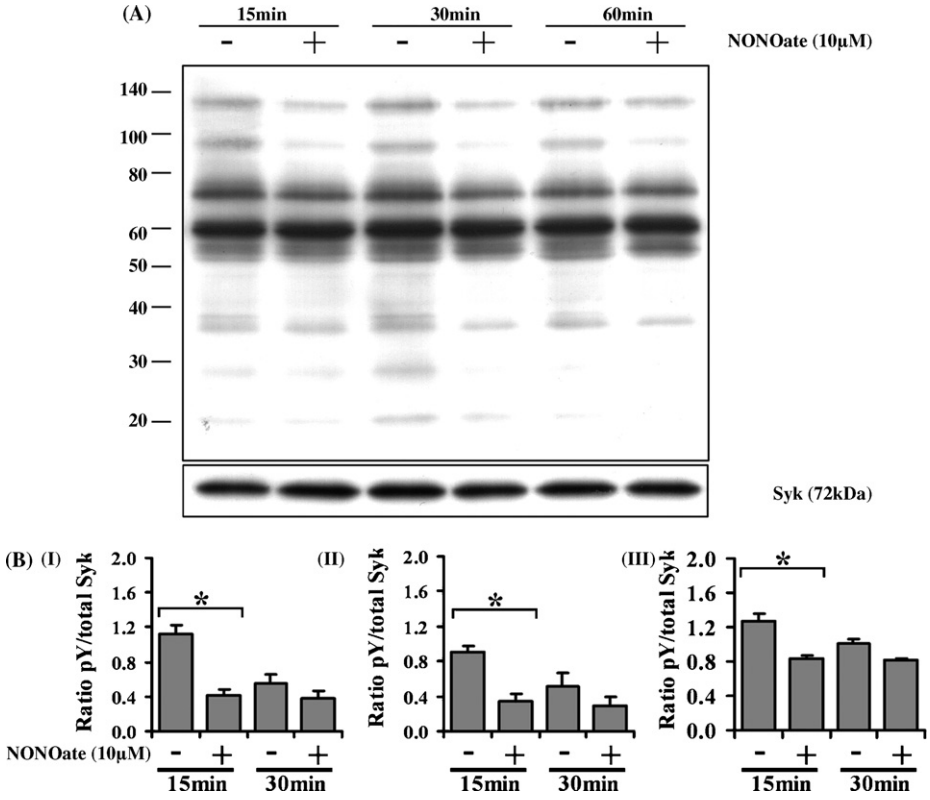

Fig. 4 – NO reduces adhesion-induced tyrosine phosphorylation of platelet proteins. (A) Washed platelets (5×10^8 platelets/ml) were allowed to adhere to fibrinogen-coated wells (six-well plates) containing ADP (10 μM) in the presence and absence of DPTA-NONOate (10 μM) for up to 60 min. At the appropriate times, cells were lysed with Laemmli buffer. After normalisation of protein content, the lysate proteins were separated by SDS-PAGE (10–18%) and immunoblotted for phosphotyrosine. Subsequently the membrane was stripped and reprobed for total Syk to check for equal loading. The blot shown is representative of three independent experiments. (B) Densitometric analysis of phosphotyrosine containing protein bands in ratio to the total Syk of selected bands (I) 125 kDa, (II) 90 kDa and (III) 72 kDa at 15 and 30 min. Results are presented as mean ± S.E.M. of three independent experiments ($^*p < 0.05$ compared to ADP alone).

antagonist in combination with NO should not increase compared to the receptor antagonist alone. Inclusion of DPTA-NONOate enhanced the inhibition of adhesion by A3P5P at each time point (Fig. 5B), which was increased to 74 ± 3% in the presence of NO ($p < 0.001$ compared to effects of A3P5P alone; Fig. 5B). Similar additive effects were observed at 60 min. Since the remaining adhesion is driven by $P2Y_{12}$ signalling our data suggest that NO can inhibit adhesion independently of $P2Y_1$-mediated Ca^{2+} mobilisation. Furthermore, ODQ failed to affect NO mediated inhibition of $P2Y_{12}$-stimulated adhesion (Fig. 5C). Thus, our data show that NO can inhibit adhesion by mechanisms that are at least partially independent of G_q-coupled $P2Y_1$ Ca^{2+} mobilisation, and do not require cGMP-mediated signalling.

We next used dimethyl-BAPTA to reduce intracellular Ca^{2+} or wortmannin to block PI3kinase activity. Adhesion to fibrinogen was completely abolished when dimethyl BAPTA (Ca^{2+}-regulated pathway) and wortmannin (PI3kinase) were used in combination (Fig. 5D), indicating that ADP induced adhesion to fibrinogen occurs through both Ca^{2+} and PI3kinase dependent pathways. Next the effects of NO on these individual pathways were investigated. Adhesion to fibrinogen was significantly, but not completely inhibited by the presence of dimethyl-BAPTA alone. The Ca^{2+} chelator caused 61 ± 7% inhibition after 15 min, demonstrating that Ca^{2+} mobilisation is required for adhesion to occur, although a significant proportion is independent of elevated Ca^{2+} (Fig. 5D). When DPTA-NONOate was used in combination with dimethyl-BAPTA, the level of inhibition at 15 min significantly increased from 61 ± 7 to 90 ± 2% ($p < 0.05$), clearly demonstrating that NO can inhibit adhesion by Ca^{2+}-independent mechanisms. Inhibition of PI3kinase with wortmannin, at concentrations previously shown to inhibit PKB phosphorylation [32], inhibited adhesion by 66 ± 3% at 15 min ($p < 0.001$). Incubation of platelets with NO in the presence of wortmannin increased inhibition from 66 ± 3 to 87 ± 1% at 15 min ($p < 0.05$), indicating that NO can inhibit adhesion in a PI3kinase-independent manner, although the combined effect was less than additive (Fig. 5D). Our data demonstrate that Ca^{2+} and PI3kinase regulated pathways independently contribute to ADP stimulated adhesion to fibrinogen. Furthermore, since adhesion requires Ca^{2+} and PI3kinase regulated pathways, and NO increases inhibition of adhesion when either pathway is blocked, our data suggest that NO inhibits both pathways independently of each other.

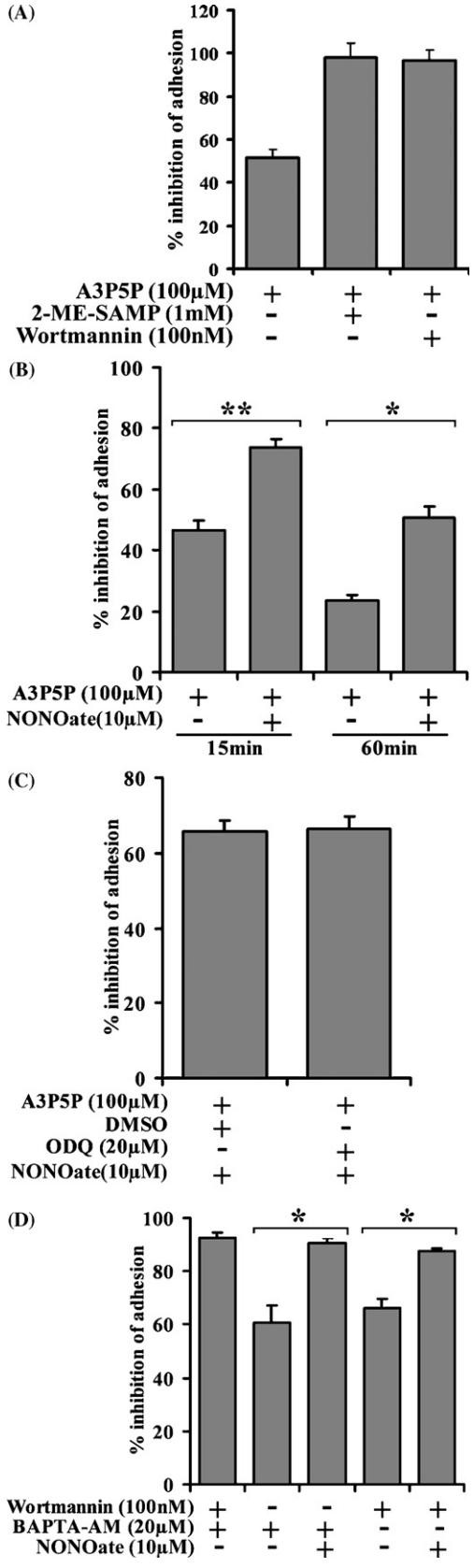

Fig. 5 – ADP stimulation of $P2Y_{12}$ induces adhesion to fibrinogen independently of $P2Y_1$. (A) Platelets were incubated with either A3P5P (100 μM) alone or in combination with either 2ME-SAMP (1 mM) or wortmannin (100 nM) before addition to fibrinogen coated wells containing ADP (10 μM) and adhesion measured after 15 incubation ($^{*}p < 0.05$, $^{**}p < 0.001$). (B) Platelets were incubated with either A3P5P (100 μM) alone or in combination with DPTA-NONOate (10 μM) before addition to fibrinogen coated wells containing ADP (10 μM). Adhesion was measured at 15 and 60 min incubation ($^{*}p < 0.05$, $^{**}p < 0.001$). (C) Experiments were performed as in (B) except that in some cases platelets were incubated with ODQ (20 μM). (D) Experiments were performed using platelets incubated with either dimethyl BAPTA or wortmannin, or both inhibitors in the presence and absence of DPTA-NONOate (10 μM) before addition to fibrinogen-coated wells containing ADP (10 μM). The data are expressed as percentage inhibition of adhesion as compared to adhesion in the absence of inhibitors at each time point. Results are presented as mean ± S.E.M. of nine independent experiments each performed in triplicate.

4. Discussion

In the present study, we have demonstrated that NO derived from the NO-donor DPTA-NONOate induced a sustained inhibition of ADP-induced adhesion under static conditions. Using the published half-life for DPTA-NONOate, the amounts of NO released from 10 μM with first order kinetics can be estimated in the region of 40 nmol/min, demonstrating that NO inhibited adhesion at physiologically relevant concentrations. Interestingly, complete inhibition of adhesion was never attained regardless of time of incubation or concentration of NO used. Thus, a significant proportion of the adhesion response to immobilised fibrinogen is resistant to NO even at high concentrations. The reasons for these findings are unclear, since ADP-induced aggregation and fibrinogen binding was completely inhibited at these concentrations of NO [33,34]. It is not due to reduced NO bioavailability, as VASP phosphorylation in adherent platelets in response to DPTA-NONOate, was maintained for up to 60 min and thus, the NO/cGMP/PKG signalling pathway was active throughout the experimental time course. It is possible that the combination of ADP-induced (inside-out) and adhesion-induced (outside-in) signalling generated by adherent platelets under static conditions is more resistant to the actions of NO compared to stimulation of platelets in suspension. Fluorescent microscopy suggested that inhibition of adhesion by NO is associated with reduced platelet spreading. Since spreading is required for stable adhesion it is possible that NO blocks spreading leading to reduced adhesion. NO-mediated inhibition of platelet adhesion and aggregation is thought to be primarily cGMP-dependent [5,35–38], although NO also inhibits platelet aggregation by cGMP-independent mechanisms [8–10]. We now extend these observations by demonstrating that NO has the capacity to inhibit adhesion to immobilised fibrinogen through a cGMP-independent mechanism, since blocking sGC with ODQ had no effect on NO-mediated inhibition of adhesion. For these interpretations to be convincing it is important to firstly confirm the NO/cGMP signalling pathway is active, and secondly that ODQ is performing as an effective inhibitor. To demonstrate both

scenarios were valid in our experimental approach we assessed the phosphorylation of VASP at serine^{239} [26]. ODQ reduced VASP phosphorylation to near basal levels but did not affect inhibition of adhesion. Thus, when NO/cGMP/PKG signalling is inactive the capacity of NO to inhibit adhesion to immobilised fibrinogen is unaffected. Indeed, the sustained VASP phosphorylation is indicative of bioactive NO throughout the experimental time course. The precise mechanism of cGMP-independent inhibition of adhesion remains elusive although it may involve PKA, nitrosylation or nitration of key proteins [7,24,39]. Under our conditions, NO clearly signals through PKA, but PKA is not involved in the inhibitory actions of DPTA-NONOate since the PKA inhibitors KT5720 and Rp-cAMP in combination, failed to affect adhesion. It has been suggested that NO-mediated inhibition of thrombin-stimulated adhesion to fibrinogen required cGMP at low NO concentrations, but involved nitration of α-actinin at higher concentrations [24]. We have previously demonstrated the presence of nitrated proteins in collagen-stimulated suspended platelets both in the presence and absence of exogenous NO [40], but were unable to detect nitrated proteins in adherent platelets under our conditions (results not shown).

Biochemical studies in suspended platelets indicate that Ca^{2+} mobilisation and PI3kinase represent targets for NO signalling, but these targets have never been studied in combination or in the physiological context of ADP-induced adhesion. Since platelet adhesion activates overlapping but also distinct signalling cascades to those in suspended platelets, it is possible that NO influences different signalling systems in suspended and adherent platelets. Our first approach was to dissect the effects of NO on Ca^{2+} and PI3kinase signalling from individual ADP receptors. Full platelet response to ADP requires both G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ [28]. In the presence of the $P2Y_1$ inhibitor, A3P5P, ADP continued to stimulate activation of $\alpha_{IIb}\beta_3$ although it was significantly reduced, indicating that $P2Y_{12}$ driven signalling is sufficient to stimulate integrin $\alpha_{IIb}\beta_3$ activation, but both receptors are required for the full response (Fig. 5), consistent with models for platelet adhesion to fibrinogen under flow [41]. Since $P2Y_1$ and $P2Y_{12}$ stimulate distinct pathways such that $P2Y_1$ activates Ca^{2+} mobilisation via G_q and $P2Y_{12}$ activates PI3kinase and inhibits adenylyl cyclase via G_i [29], it suggests a PI3kinase-dependent but Ca^{2+}-independent adhesion. It is proposed that NO inhibits platelet aggregation by reducing Ca^{2+}-mobilisation in response to agonists. Hence we wished to investigate the possibility that NO could block $P2Y_{12}$-mediated adhesion which is primarily Ca^{2+} independent. NO inhibited $P2Y_{12}$-mediated adhesion to fibrinogen, thus providing novel evidence that NO targets G_i coupled signalling in platelets, and suggesting that NO could inhibit adhesion independently of blocking increases in intracellular Ca^{2+}. To further explore this possibility we used the cell permeable Ca^{2+} chelator dimethyl-BAPTA. ADP stimulated $\alpha_{IIb}\beta_3$-mediated platelet adhesion occurred in the presence of dimethyl BAPTA, although it was significantly reduced compared to ADP alone. These data are consistent with the A3P5P experiments since they indicate that Ca^{2+} mobilisation is essential for full $\alpha_{IIb}\beta_3$ activation, but alternate pathways leading to partial integrin activation do exist. ADP also stimulates PI3kinase activity through $P2Y_{12}/G_i$-signalling. Consistent with a role for PI3kinase activity in ADP induced activation of $\alpha_{IIb}\beta_3$, wortmannin significantly but not completely reduced adhesion to fibrinogen. Simultaneous inhibition of both pathways abolished adhesion. Thus, ADP induced $\alpha_{IIb}\beta_3$ activation and adhesion to fibrinogen can occur through either Ca^{2+} or PI3kinase dependent pathways. When platelets were incubated with NO in the presence of dimethyl BAPTA, NO increased adhesion to a greater extent than BAPTA alone, clearly indicating that NO regulates platelet adhesion through Ca^{2+} independent mechanisms under these conditions. Since the adhesion remaining in the presence of dimethyl BAPTA is PI3kinase-dependent, it is likely that NO is targeting the PI3kinase pathway. This is consistent with the finding of Pigazzi et al. who first demonstrated that NO directly inhibits PI3kinase activity in suspended platelets [21]. Additionally, in our study NO increased inhibition of adhesion by wortmannin suggesting actions independent of PI3kinase most likely on Ca^{2+} mobilisation as observed in suspended platelets [15–17]. The nature of the cGMP-independent mechanism regulating platelet adhesion requires further investigation.

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