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cGMP-independent inhibition of integrin $\alpha_{IIb}\beta_3$-mediated platelet adhesion and outside-in signalling by nitric oxide

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Abstract **We examined the influence of *S*-nitrosoglutathione (GSNO) on $\alpha_{IIb}\beta_3$ integrin-mediated platelet adhesion to immobilised fibrinogen. GSNO induced a time- and concentration-dependent inhibition of platelet adhesion. Inhibition was cGMP-independent and associated with both reduced platelet spreading and protein tyrosine phosphorylation. To investigate the cGMP-independent effects of NO we evaluated integrin β_3 phosphorylation. Adhesion to fibrinogen induced rapid phosphorylation of β_3 on tyrosines 773 and 785, which was reduced by GSNO in a cGMP independent manner. Similar results were observed in suspended platelets indicating that NO-induced effects were independent of spreading-induced signalling. This is the first demonstration that NO directly regulates integrin β_3 phosphorylation.**

Keywords: Nitric oxide; Platelets; cGMP; Integrin $\alpha_{IIb}\beta_3$; Tyrosine phosphorylation

1. Introduction

The integrin $\alpha_{IIb}\beta_3$ mediates platelet adhesion to immobilised fibrinogen. $\alpha_{IIb}\beta_3$ is found on the platelet surface in a cryptic low affinity form, but upon inside-out signalling induced by platelet agonists, it is converted to its high affinity form capable of ligand binding. $\alpha_{IIb}\beta_3$ is targeted by intracellular signalling cascades, but 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 von Willebrand Factor or fibrinogen surfaces and their resistance to detachment [1,2]. The cytoplasmic tail of the β-integrin is considered critical to integrin-mediated platelet spreading through interactions with the cytoskeleton [3,4]. Ligand-induced phosphorylation of β_3-integrin leads to its interaction with signalling proteins such as the Src family kinases, Syk, protein kinase C, phospholipase Cγ2 and with structural cytoskeletal proteins such as α-actinin and talin [2–5]. Although a model for the activation of outside-in signalling pathways is emerging, little is known of the negative regulation of integrin signalling.

Nitric oxide (NO) acts to both inhibit platelet adhesion to the extra cellular matrix (ECM) and limit platelet aggregation [6,7]. NO blunts agonist-induced biochemical signalling in suspended platelets leading to reduced integrin activation, Ca^{2+} mobilisation, secretion and thromboxane A_2 (TxA_2) release [8]. While it is established that NO inhibits inside-out signalling and $\alpha_{IIb}\beta_3$ activation induced by agonists [9], it is unclear if NO inhibits outside-in signalling mediated through β_3 phosphorylation. We examined the effects of NO on outside-in signalling using platelet adhesion and β_3 phosphorylation as markers of integrin function. Our data demonstrate that NO reduces integrin-mediated signalling in a cGMP-independent manner, via modulation of β_3 phosphorylation suggesting a novel mechanism of NO action in platelet signalling.

2. Materials and methods

2.1. Materials

The antibodies used were anti-phosphotyrosine monoclonal antibody (MoAb) 4G10 (Upstate Biotechnology Inc., Milton Keynes, UK), anti-Syk mouse MoAb (Santa Cruz, Herts., UK), phospho-Ser 239 VASP and anti-integrin β_3 antibodies (Cell Signalling Technology, Hitchin, UK), phospho-Tyr 773 phospho-Tyr 785 integrin β_3 (Biosource Intern., CA, USA), anti-mouse and anti-rabbit IgG HRP (Amersham Biosciences, Bucks., UK). DC protein assay kit was purchased from Bio-Rad (Hemel-Hempstead, Herts., UK). 1H-[1,2,4]-Oxadiazolo [4, 3-a]-quinoxalin-1-one (ODQ) was obtained from Calbiochem (Nottingham, UK). All other reagents were from Sigma Ltd (Poole, UK).

2.2. Platelet preparation

Human blood was taken from drug-free volunteers using acid citrate dextrose (29.9 mM Na citrate, 113.8 mM glucose, 72.6 mM NaCl and 2.9 mM citric acid, pH 6.4) as anticoagulant. Washed platelets were prepared by the prostaglandin method [10] and were incubated with apyrase and indomethacin (10 μM) to abrogate the effects of ADP, and $T \times A_2$ respectively.

2.3. Platelet adhesion assay

96-well micro titre plates were coated using fibrinogen (1 mg/ml) or heat-inactivated human serum (HS) for 24 h at 4 °C and blocked with HS (5%) diluted in PBS. Platelets were incubated with inhibitors for the required time and then allowed to adhere at 37 °C for the indicated time. Non-adherent platelets were removed and 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

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X-100, pH 5.4) for 1 h at room temperature. The reaction product was visualised using 2 N NaOH at 405 nm [11]. None of the platelet reagents used had any effect on platelet acid phosphatase activity (not shown).

2.4. Microscopy

Platelets (2×10^7/ml) were adhered to fibrinogen (1 mg/ml)-coated coverslips for 30 min at 37 °C. In the presence and absence of GSNO (100 μM). Non-adherent platelets were removed, and adherent platelets were fixed with 4% paraformaldehyde and permeablised with 0.1% Triton-X-100. 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 and the results calculated as mean number of adherent platelet ± S.E.M. per 0.1 mm^2.

2.5. Immunoblotting studies

Platelets (5×10^8/ml) were adhered to fibrinogen (1 mg/ml)-coated wells (six-well plates) in the presence or absence of GSNO for up to 60 min. Non-adherent platelets were removed and adherent platelets lysed by the addition of Laemmli sample buffer (1×). For VASP studies both adherent and non-adherent platelets were immunoblotted. After accounting for fibrinogen, lysate protein (20 μg) was subjected to SDS–PAGE (10–18%) and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with either anti-phosphotyrosine 4G10 (1:1000), anti-phospho-β_3Y^{773}, anti-phospho-β_3Y^{785} (1:1000) or anti phosphoserine^{239} VASP (1:1000), and stripped and reprobed with either anti-Syk (1:2000), anti-β_3 or anti-VASP. All antibodies were diluted in TBS-T containing 2% BSA Membranes were visualised with enhanced chemiluminescence (ECL). Densitometric analyses were performed using Scion Image program (NIH), and expressed as a ratio of phosphotyrosine to total Syk or total β_3. Protein concentrations were determined in platelet lysates using the BioRad protein assay using bovine serum albumin as standard.

2.6. 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.

3. Results

3.1. The effect of exogenous NO on integrin $\alpha_{IIb}\beta_3$-mediated platelet adhesion

Incubation of platelets in fibrinogen coated wells in the absence of platelets agonists led to a significant increase in platelet adhesion. This response was $\alpha_{IIb}\beta_3$-dependent since preincubation of platelets with Arg-Gly-Asp-Ser peptide (RDGS; 2 mM), an $\alpha_{IIb}\beta_3$ peptide blocker, ablated adhesion ($P < 0.001$; Fig. 1). Incubation with GSNO led to a concentration-dependent inhibition of platelet adhesion to immobilised fibrinogen (ANOVA: $P \leqslant 0.001$) (Fig. 2A). After 15 min, adhesion was inhibited by 16.5 ± 8.4% ($P \leqslant 0.05$) using 1 μM GSNO, and maximal effects observed at 100 μM GSNO (41.5 ± 4.6%, $P \leqslant 0.001$) (Fig. 2A, black fill). Similar results were obtained with a structurally distinct NO donor, DPTA-NONOate, while NO-depleted GSNO had no effect (not shown).

GSNO inhibits platelet aggregation through cGMP-dependent and independent mechanisms [12,13], however it is unknown if the same is true for integrin-driven adhesion. To address this issue we used the soluble guanylyl cyclase (sGC) inhibitor ODQ [14]. GSNO (100 μM) inhibited platelet adhesion by 41.5 ± 4.6%, which surprisingly was unaffected by the presence ODQ (20 μM) (43.8 ± 5.2% Fig. 2A), suggesting that integrin $\alpha_{IIb}\beta_3$-mediated outside-in signalling and adhesion is inhibited by GSNO in a sGC-independent manner.

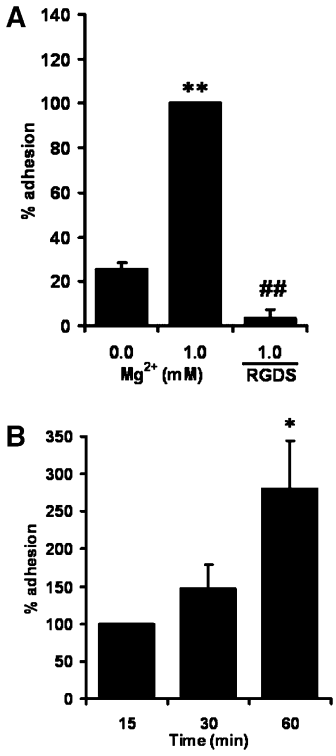

Fig. 1. Platelet adhesion in the absence of exogenous agonists and secondary mediators. (A) Washed platelets (1×10^8 platelets/ml) were suspended in Mg^{2+}-free or Mg^{2+} (1 mM)-replete Tyrode's buffer were adhered to fibrinogen (1 mg/ml) coated 96-well plates. Platelets were incubated for 15 min at 37 °C in the presence of apyrase (1U/ml), indomethacin (10 μM), and RGDS (2 mM) as indicated. Adhesion was measured spectrophotometrically as described in experimental procedures. Results are presented as means ± S.E.M. of five independent experiments each performed in triplicate. ($^{**}P \leqslant 0.001$ compared to no Mg^{2+}; $^{\#\#}P \leqslant 0.001$ compared to the absence of RGDS). (B) Washed platelets were prepared as in (A) and left to adhere for 15, 30, and 60 min ($^{*}P < 0.05$ compared to 15 min). Adhesion in the presence of 1 mM Mg^{2+} after 15 min was regarded as 100%.

ODQ ablated NO-induced sGC activity as evidenced by the inhibition of VASP phosphorylation at serine^{239}, as a marker of the NO/sGC/Protein kinase G (PKG) signalling pathway [15]. Furthermore, ODQ completely inhibited GSNO-induced cGMP formation in suspended platelets (not shown). Thus, NO-mediated inhibition of adhesion under these conditions occurs via a cGMP-independent mechanism. The platelet adhesion assay results were confirmed by staining of the actin cytoskeleton using TRITC-labelled phalloidin. Platelets adhered to fibrinogen and underwent the full range of morphological changes from filopodia formation to full spreading (Fig. 2C and D). The number of platelets forming stable adhesions on fibrinogen was 182 ± 12 per 0.1 mm^2, which was reduced to 114.0 ± 1 platelets/0.1 mm^2 in the presence of GSNO(10 μM) (Fig. 2C and D), representing a 36% reduction in platelet adhesion ($P \leqslant 0.01$). Furthermore, GSNO was able to inhibit the ability of adherent platelets to spread (Fig. 2D).

3.2. Effect of NO on integrin $\alpha_{IIb}\beta_3$-mediated outside-in signalling

β_3 phosphorylation is critical to facilitating the platelet adhesion response through initiation of signalling cascades that

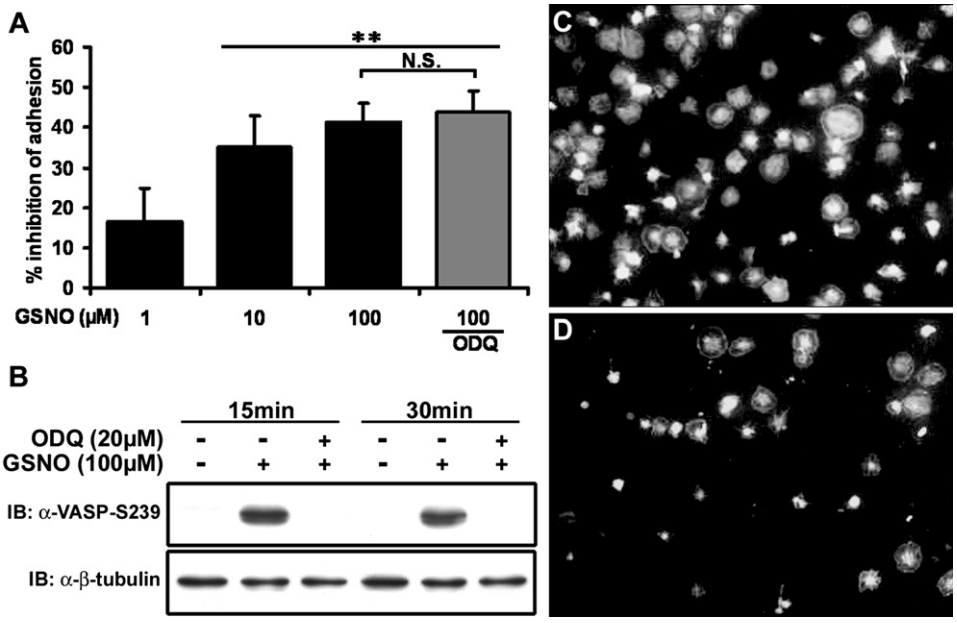

Fig. 2. NO-mediated inhibition of adhesion to immobilised fibrinogen. (A) Washed platelets (1×10^8 platelets/ml) were preincubated for 10 min with GSNO (0–100 μM), and ODQ (20 μM) as indicated, and allowed to adhere to fibrinogen-coated wells at 37 °C for 15 min ($^{**}P < 0.001$ compared to no GSNO treatment). (B) Washed platelets (5×10^8 platelets/ml) were preincubated with GSNO and added to fibrinogen-coated wells (6-well plates) in the presence and absence of ODQ (20 μM) for up to 30 min. Cells were lysed, separated by SDS–PAGE and immunoblotted for phosphoVASP-serine[239] and reprobed for β-tubulin. The blot is representative of three independent experiments. (C) and (D) Platelets were adhered to fibrinogen-coated coverslips for 15 min at 37 °C in absence (C) and presence (D) of GSNO (100 μM). Adherent platelets were stained for F-actin with TRITC-conjugated phalloidin. The data are then expressed as means ± S.E.M. of three number of adherent per 0.1 mm^2 independent experiments performed in duplicate ($^{*}P \leqslant 0.05$).

lead to actin polymerisation [16,17]. Integrin $\alpha_{IIb}\beta_3$-mediated adhesion to immobilised fibrinogen for 15 min induced tyrosine phosphorylation in a number of proteins, with robust phosphorylation observed in proteins apparent molecular weights of approximately 125 kDa, 90 kDa, 72 kDa 55 kDa and 39 kDa (Fig. 3A). Beyond this time point adhesion induced tyrosine phosphorylation decreased (Fig. 3B). In the presence of GSNO tyrosine phosphorylation of a number of proteins was inhibited, with the most notable reduction was observed at 15mins with the 90 kDa protein band ($P \leqslant 0.05$; Fig. 3B).

3.3. Effect of NO on individual integrin$\beta_3$phosphorylation sites

The integrin β_3 becomes phosphorylated on tyrosine[773] and tyrosine[785] (human sequence) in response to ligand binding [18] (Fig. 4A). Since the 90 kDa phosphoprotein could represent integrin β_3 (Fig. 4A), we investigated whether both residues become phosphorylated during platelet adhesion and whether NO exhibits its inhibitory effect in a tyrosine residue specific manner. Immunoblotting of proteins from adherent platelets or suspended platelets treated with fibrinogen demonstrated increased phosphorylation of tyrosine[773/783] (Fig. 4B), which was inhibited significantly by GSNO (100 μM) ($P \leqslant 0.05$). Since NO inhibits platelet adhesion and spreading, the reduced tyrosine phosphorylation of β_3 may represent a reduced spreading induced signalling. To obviate spreading induced signalling the experiments were repeated in suspended platelets using soluble fibrinogen (250 μg/ml) in the presence of 1 mM Mn^{2+} [16]. Under these conditions β_3 phosphorylation at tyrosine[773/783] was increased and consistent with adhesion experiments was inhibited significantly by GSNO (100 μM) (Fig. 4C). When platelets were exposed to NO

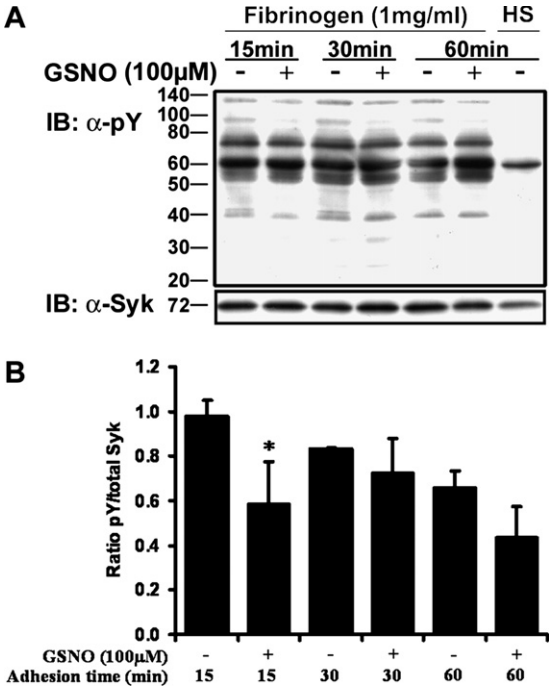

Fig. 3. NO reduces adhesion induced tyrosine phosphorylation of platelet proteins. (A) Washed platelets (5×10^8 platelets/ml) were adhered to fibrinogen-coated wells (6-well plates) in the presence and absence of GSNO (100 μM) for up to 60 min at 37 °C. In control experiments platelets were also incubated in wells coated with heat-inactivated human serum (HS). At the appropriate times, cells were lysed, the lysate proteins were separated by SDS–PAGE (10–18%) and immunoblotted for phosphotyrosine. The blot shown is representative of three independent experiments. (B) Densitometry measurement of the 90 kDa band expressed as ratio of pY/total Syk and presented as means ± S.E.M. of three independent experiments ($^{*}P \leqslant 0.05$).

(100 μM GSNO) in the presence and absence of ODQ, tyrosine phosphorylation of the integrin remained inhibited (Fig. 4D, lanes 1 and 4).

4. Discussion

Platelet adhesion and spreading on immobilised fibrinogen occurs in the absence of exogenously added platelet agonists [19]. We used this phenomenon to study the effects NO on integrin signalling. NO induced a sustained inhibition of adhesion, which was concentration dependent, but also reduced platelet spreading on fibrinogen. Platelet spreading is required for stable adhesion and it is possible that NO blocks spreading leading to reduced adhesion. 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 this are unclear since GSNO at these concentrations completely inhibits agonist-induced aggregation and fibrinogen binding [9,20]. These high concentrations of NO are associated with cGMP independent effects on platelets. Certainly the kinetics of the adhesion assay would suggest that cGMP levels, which peak approximately 1 min after exposure to NO [21], would have return to basal before significant adhesion had taken place

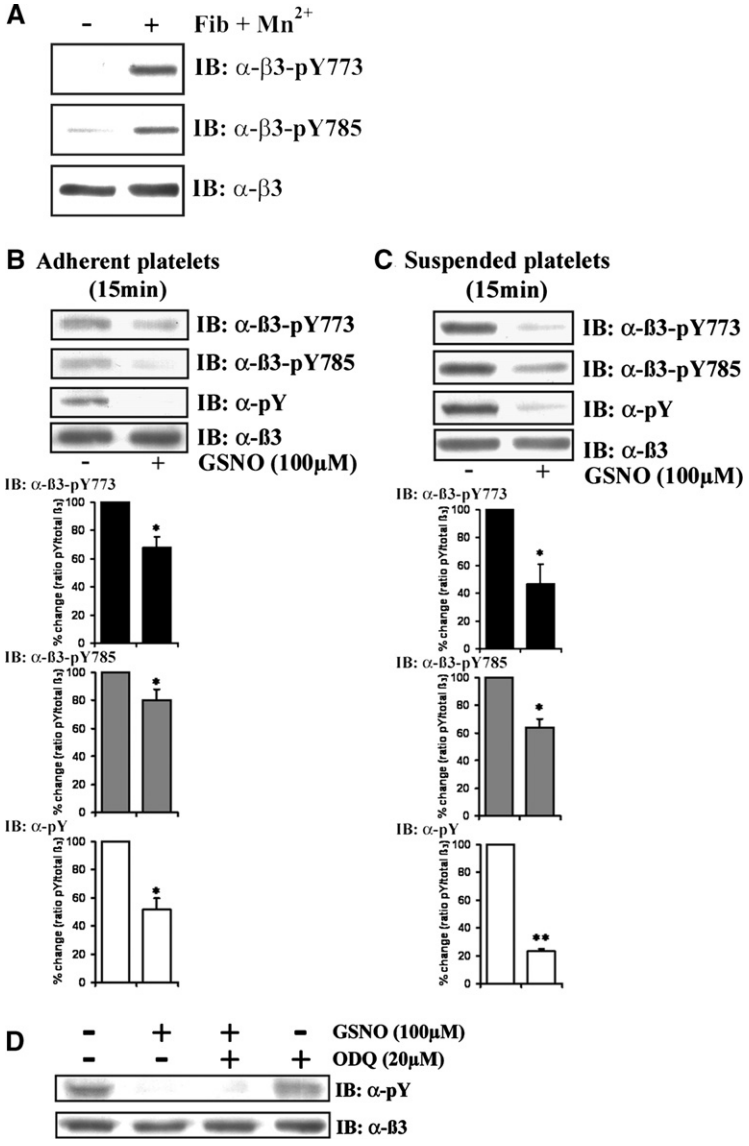

Fig. 4. Effect of NO on phosphorylation of cytoplasmic tyrosine residues in adherent platelets. (A) Washed platelets (3×10^8 platelets/ml) were incubated with in the presence (lane 2) and absence (lane 1) fibrinogen (250 μg/ml)/Mn^{2+} (1 mM) 37 °C for 15 min. (B) Washed platelets (5×10^8 platelets/ml) were adhered to fibrinogen-coated wells (6-well plates) in the presence and absence of GSNO for 15 min at 37 °C. (C) Washed platelets (3×10^8 platelets/ml) were incubated with fibrinogen (250 μg/ml) in the presence of 1 mM Mn^{2+} 37 °C for 15 min. Cells were lysed and processed as in (A). (D) Platelets were treated as in (C) except that in some cases the platelets were preincubated with ODQ (20 μM). In all cases cells were lysed with Laemmli buffer and after normalisation of protein content, the lysate proteins were separated by SDS–PAGE (10–18%) immunoblotted for phosphotyrosine, phospho-β_3Y^{773}, and phospho-β_3Y^{785}. Membranes were subsequently stripped and reprobed with a total β_3 antibody. Densitometry analysis was conducted on integrin β_3-pY^{773}, integrin β_3-pY^{785}, and total integrin β_3-pY. Results represent the means ± S.E.M. of four independent experiments expressed as percentage change, calculated using the ratio of the according pY/total β_3. Ratio obtained in the absence of NO treatment was considered 100% ($^*P \leqslant 0.05$ and $^{**}P \leqslant 0.001$ compared to no GSNO treatment).

favouring a cGMP independent mechanism. Indeed, NO-mediated platelet adhesion was cGMP-independent despite activation of the PKG signalling pathway. It is possible that cGMP signalling is important for short term regulation of platelets, but prolonged inhibition is mediated by cGMP-independent mechanisms. Furthermore adhesion-induced (outside-in) signalling generated by adherent platelets under static conditions is more resistant to the actions of NO, whether they are cGMP-dependent or independent, compared to stimulation of platelets in suspension.

Platelet adhesion and spreading on fibrinogen activates protein tyrosine phosphorylation signalling cascades [5,22]. We observed robust phosphorylation of proteins of apparent molecular weights of 125 kDa, 90 kDa, 72 kDa, 55 kDa and 39 kDa. Interestingly NO specifically reduced phosphorylation of the 90 kDa protein band. Since $\beta_3$integrin has an apparent molecular weight of 90 kDa, we tested the effects of NO on β_3 phosphorylation. Platelet adhesion was associated with β_3 phosphorylation on two residues, tyrosine 773 and 785. Importantly the level of phosphorylation was reduced by the presence of GSNO. Importantly, GSNO also inhibited tyrosine phosphorylation of β_3 induced by soluble fibrinogen in the presence of Mn^{2+}, indicating that NO inhibited phosphorylation directly by reducing spreading-induced signalling. Thus, NO inhibits β_3 phosphorylation, which is associated with a reduced adhesion and spreading response.

The precise cGMP-independent mechanism by which NO regulates integrin function remains elusive. Possible mechanisms of action for NO, independent of soluble guanylyl cyclase include protein *S*-nitrosylation and nitration. The integrin $\alpha_{IIb}\beta_3$ has an extracellular cysteine-rich domain in the β_3 subunit that includes a number of unpaired cysteines. Rearrangement of disulfide bonds in an enzyme dependent process is required in order to allow fibrinogen binding to the integrin [23–25]. NO can induce *S*-nitrosylation of cysteine residues in a number of proteins leading to altered function. *S*-nitrosylation of β_3 by NO would prevent sulfhydryl rearrangement within the integrin and render the integrin either unable to engage with fibrinogen effectively or affect its ability to transduce signals. An alternative mechanism may involve protein disulphide isomerase (PDI), an enzyme that rearranges disulphide bonds, which is present on the platelet surface and has been implicated in facilitating integrin activation [23]. A recent study by Uehara et al. demonstrated the inhibition of PDI by *S*-nitrosylation in cerebrocortical neurons [26]. If NO inhibits platelet PDI this could result in failure to facilitate integrin activation. Certainly a mechanism involving *S*-nitrosylation is consistent with the higher concentrations of NO required in inhibit integrin function, since sGC activation occurs at much lower concentrations [27]. In conclusion our data demonstrate for the first time that NO inhibits β_3 phosphorylation, integrin-mediated outside-in signalling leading to reduced platelet adhesion, in a cGMP-independent manner.

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