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A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations

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1 Nitric oxide (NO) is a potent inhibitor of platelet activation, that inhibits the agonist-induced increase in cytosolic Ca^{2+} concentration through both cGMP-dependent and independent pathways. However, the NO-related (NO_x) species responsible for cGMP-independent signalling in platelets is unclear. We tested the hypothesis that extracellular NO, but not NO^+ or peroxynitrite, generated in the extracellular compartment is responsible for cGMP-independent inhibition of platelet activation *via* inhibition of Ca^{2+} signalling.

2 Concentration–response curves for diethylamine diazeniumdiolate (DEA/NO; a spontaneous NO generator), *S*-nitroso-*N*-valerylpenicillamine (SNVP; an *S*-nitrosothiol) and 3-morpholinosydnonomine (SIN-1; a peroxynitrite generator) were generated in platelet-rich plasma (PRP) and washed platelets (WP) in the presence and absence of a supramaximal concentration of the soluble guanylate cyclase inhibitor, ODQ (20 μM). All three NO_x donors displayed cGMP-independent inhibition of platelet aggregation in PRP, but only DEA/NO exhibited cGMP-independent inhibition of aggregation in WP.

3 Analysis of NO generation using an isolated NO-electrode revealed that cGMP-independent effects coincided with the generation of substantial levels of extracellular NO (>40 nM) from the NO_x donors.

4 Reconstitution of WP with plasma factors indicated that the copper-containing plasma protein, caeruloplasmin (CP), catalysed the release of NO from SNVP, while Cu/Zn superoxide dismutase (SOD) unmasked NO generated from SIN-1. The increased generation of extracellular NO correlated with a switch to cGMP-independent effects with both NO_x donors.

5 Analysis of Fura-2 loaded WP revealed that only DEA/NO inhibited Ca^{2+} signalling in platelets *via* a cGMP-independent mechanism. However, preincubation of SNVP and SIN-1 with CP and SOD, respectively, induced cGMP-independent inhibition of intraplatelet Ca^{2+} trafficking by the NO_x donors.

6 Taken together, our data suggest that extracellular NO (>40 nM) is required for cGMP-independent inhibition of platelet activation. Plasma constituents may play an important pharmacological role in activating cGMP-independent signalling by *S*-nitrosothiols or peroxynitrite generators.

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Abbreviations: Asc, ascorbate; AUC, area under curve; cGMP, cyclic guanosine-3′5′-monophosphate; CP, caeruloplasmin; DEA/NO, (Z)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate sodium salt; DTS, dense tubular system; HSA, human serum albumin; IBMX, 3-Isobutyl-1-methyl xanthine; NOS, nitric oxide synthase; ODQ, 1-*H*-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one; PGI_2, prostacyclin; PRP, platelet-rich plasma; sGC, soluble guanylate cyclase; SIN-1, 3-morpholinosydnonomine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNVP, *S*-nitroso-*N*-valerylpenicillamine; SOD, superoxide dismutase; TxA_2, thromboxane A_2; WP, washed platelets

Introduction

Nitric oxide (NO) is a labile-free radical mediator that potently inhibits platelet aggregation and adhesion to the vascular endothelium (Radomski *et al*., 1987a–c; Pigazzi *et al*., 1999). NO is synthesized by constitutive NO synthase in the endothelium (Palmer *et al*., 1987; 1988) and in platelets themselves (Radomski *et al*., 1990), and has long been recognized to inhibit platelet activation by increasing the synthesis of cyclic-3′5′-guanosine monophosphate (cGMP) *via* direct stimulation of the enzyme soluble guanylate cyclase (sGC; Mcdonald & Murad, 1995). Activation of G-kinase by cGMP inhibits platelet function through phosphorylation of key proteins such as thromboxane A_2 (TxA_2) receptors (Wang *et al*., 1998) and proteins involved in the Ca^{2+} signalling

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pathway (Kawahara *et al*., 1984; Nakashima *et al*., 1986; Busse *et al*., 1987; Matsuoka *et al*., 1989; Mcdonald & Murad, 1995; Cavallini *et al*., 1996). An elevated cytosolic Ca^{2+} concentration following agonist stimulation is a critical signalling event required for platelet shape change and aggregation (Gerrard *et al*., 1978; Murer, 1985; Rink, 1988; Blockmans *et al*., 1995).

Recently, several cGMP independent signalling mechanisms have been identified (Gordge *et al*., 1998; Trepakova *et al*., 1999; Tsikas *et al*., 1999; Ahern *et al*., 2002; Homer & Wanstall, 2002; Thyagarajan *et al*., 2002; White *et al*., 2002). In platelets, NO accelerates sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)-dependent refilling of internal Ca^{2+} stores (Trepakova *et al*., 1999; Homer & Wanstall, 2002), and the unstable *S*-nitrosothiol, *S*-nitrosocysteine, inhibits agonist-induced TxA_2 synthesis in human platelets (Tsikas *et al*., 1999). Furthermore, the importance of cGMP-independent mechanisms is underpinned by the recent discovery that G-kinase has an excitatory role in platelet activation (Li *et al*., 2003).

An important aspect in assessing cGMP-independent mechanisms is identification of the exact NO-related (NO_x) species responsible for the effect. However, studies in platelets are complicated by a number of factors. Firstly, blood plasma contains antioxidants such as ascorbate (Asc; $\sim 100\,\mu$M; Esteve *et al*., 1997) and low molecular weight thiols (10–20 μM; Mansoor *et al*., 1992), which can catalyse the release of NO from *S*-nitrosothiols (Ignarro *et al*., 1981; Singh *et al*., 1996). Secondly, NO and its higher oxides can interact with plasma proteins such as albumin and haemoglobin, resulting in the formation of *S*-nitrosated proteins with considerably different properties than NO itself (Stamler *et al*., 1992; Simon *et al*., 1993; Scharfstein *et al*., 1994; Kharitonov *et al*., 1995; Gow *et al*., 1997; 1999; Pawloski *et al*., 1998; Crane *et al*., 2002). Plasma is also an abundant source of the Fe^{2+}/Cu^{2+} transporting protein caeruloplasmin (CP), which catalyses *S*-nitrosothiol formation and decomposition (Dicks & Williams, 1996; Inoue *et al*., 1999). Thirdly, NO can react with superoxide (O_2^-) at almost diffusion limited rates, leading to the generation of peroxynitrite ($ONOO^-$; Jourd'heuil *et al*., 2001; Espey *et al*., 2002). $ONOO^-$ has been reported to exert both inhibitory and excitatory effects in platelets (Moro *et al*., 1994; Brown *et al*., 1998). In the case where the simultaneous generation of NO and O_2^- is desired to create $ONOO^-$, plasma may contain enough antioxidants to remove at least a proportion of O_2^- before it has the opportunity to react with NO. Finally, endogenous pathways exist for the conversion of $ONOO^-$ to potent nitrosating species such as N_2O_3, which can lead to formation of *S*-nitrosothiols and NO (Mayer *et al*., 1998; Espey *et al*., 2002).

To date, a number of studies in both platelets and blood vessels have indicated a correlation between the amount of NO (radical) released by an NO donor, and the level of cGMP-independent activity observed (Homer *et al*., 1999; Sogo *et al*., 2000; Miller *et al*., 2004). Our aim in these experiments was to perform systematic experiments to test the hypothesis that *extracellular* generation of NO, but not $ONOO^-$ or *S*-nitrosothiols, is the most important determinant for cGMP-independent inhibition of platelet activation. Furthermore, we hypothesized that extracellular generation of NO instills antiplatelet effects *via* inhibition of Ca^{2+} mobilization. Using the plasma proteins CP and superoxide dismutase (SOD) as membrane impermeant tools to elicit the release of NO from NO_x donors, we have probed the role of plasma antioxidants and extracellular NO in cGMP-independent inhibition of human platelet activation.

Methods

Materials

(Z)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate sodium salt (DEA/NO; Alexis Biochemicals, Nottingham, U.K.) was dissolved in 0.01 M NaOH and stored at -20°C. Immediately prior to use, DEA/NO was diluted in phosphate-buffered saline (PBS, pH 7.4). *S*-nitroso-*N*-valerylpenicillamine (SNVP) was synthesized as described (Miller *et al*., 2000), dissolved in PBS and stored at -20°C in the dark. 3-morpholinosydnonomine (SIN-1) was also dissolved in PBS and stored at -20°C. 1-*H*-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris Cookson, Langford, Bristol, U.K.) was dissolved in dimethylsulphoxide (DMSO) and stored at -20°C. All other chemicals were purchased from Sigma (Poole, Dorset, U.K.).

Platelet preparation

Venous blood was drawn from the antecubital fossa of healthy volunteers (aged 20–40 years) into citrated tubes (0.38% final concentration). Volunteers had not taken any medication known to affect platelet aggregation within the last 10 days. Platelet-rich plasma (PRP) and platelet poor plasma (PPP) were obtained from whole blood by centrifugation as previously described (Sogo *et al*., 2000). Washed platelets (WP) were derived from PRP by centrifugation in the presence of 300 ng ml^{-1} prostacyclin (PGI_2) to prevent activation, followed by resuspension in PGI_2-free HEPES-tyrode buffer containing (in mM): 137 NaCl, 2.7 KCl, 1.05 $MgSO_4$, 0.4 NaH_2PO_4, 0.8 $CaCl_2$, 12.5 $NaHCO_3$, 5.6 glucose and 10 HEPES. Although previous evidence suggests that HEPES may catalyse NO consumption *via* the release of O_2^- (Keynes *et al*., 2003), experiments performed in this laboratory indicate that HEPES primarily acts as an antioxidant in this system (data not shown; unpublished observation). The platelet count in PRP and WP was determined using a Coulter A^c.T 8 Haematology analyzer (Coulter Electronics, U.K.), and standardized to 250×10^9 l^{-1} *via* dilution with PPP (PRP) or HEPES-tyrode buffer (WP).

cGMP measurements

cGMP measurements were performed to assess the concentration of ODQ required to completely inhibit cGMP synthesis to a maximal dose of DEA/NO (10 μM). Aliquots (0.5 ml) of PRP or WP were equilibrated to 37°C in a platelet aggregometer and incubated with the phosphodiesterase inhibitor 3-Isobutyl-1-methyl xanthine (IBMX; 1 mM) for 20-min prior to the addition of DEA/NO (10 μM). To assess the inhibitory action of ODQ on cGMP formation, WP or PRP were preincubated with ODQ (20 or 100 μM) for 15-min prior to the addition of DEA/NO. In all cases, DEA/NO was incubated in the platelets for 30 s prior to the addition of 300 μl of 10% trichloroacetic acid to lyse platelets and precipitate the proteins. The 30 s time point was used so that cGMP measurement occurred shortly after peak cGMP synthesis (Bellamy *et al*., 2000). The resulting mixture was then centrifuged ($2000 \times g$; 10-min), and the

supernatant aspirated and stored (−20°C; <2 weeks) prior to cGMP ELISA (low pH, R&D systems, Abington, U.K.). The nonacetylated form of ELISA was used, and results expressed as pmol/10^8 platelets ($n=5$, with ELISA samples run in duplicate).

NO electrode measurements

NO generation was measured using an isolated NO electrode (World Precision Instruments, Stevenage, U.K.). Data were captured *via* an Apollo 4000 Free Radical Analyser (World Precision Instruments). The electrode was calibrated using DEA/NO (0.1–3.2 μM) in phosphate buffer (pH 4.0); DEA/NO decomposition is extremely rapid at pH$\leqslant$5.0 (Davies *et al*., 2001). Aliquots (2 ml) of PRP or WP were equilibrated to 37°C before the addition of the NO_x donors DEA/NO (3 μM), SNVP (100 μM) or SIN-1 (100 μM). NO concentration was then recorded for 6-min. DEA/NO is a well-characterized NO-donor that undergoes rapid hydrolysis in PBS (Davies *et al*., 2001) without a requirement for biological factors. SNVP, a more lipophilic analogue of the well-recognized *S*-nitrosothiol, *S*-nitroso-*N*-acetylpenicillamine (SNAP), was chosen for these studies because it is a relatively stable *S*-nitrosothiol, but nevertheless undergoes transnitrosation reactions (Megson *et al*., 1999), and is therefore useful in distinguishing between *S*-nitrosothiol and NO-mediated effects. SIN-1 generates O_2^- concurrently with NO (Feelisch *et al*., 1989; Noack & Feelisch, 1991), and given that the reaction rate between NO and O_2^- is near diffusion limited ($6.7\times10^9\,M^{-1}\,s^{-1}$; Huie & Padmaja 1993), SIN-1 is an effective $ONOO^-$ donor. In experiments involving sGC inhibition, PRP was treated with a supramaximal concentration of ODQ (20 μM) for 15-min as determined in the preliminary cGMP measurements (Miller *et al*., 2004) before the addition of NO_x donor. In further experiments, the effect of plasma factors on the release of NO from SNVP and SIN-1 was investigated. WP were incubated with levels of CP that approximate plasma concentrations (0.4 g l^{-1}; Ravin, 1961; Prakasam *et al*., 2001) for 1-min prior to the addition of SNVP (100 μM). Similarly, WP was incubated with SOD (500 U ml^{-1}), Asc (100 μM), human serum albumin (HSA, 4%) for 1-min prior to the addition of SIN-1 (100 μM; $n=4$ for all experiments).

Aggregometry

Aggregometry was carried out using a four-channel platelet aggregometer (Chronolog 470 VS, Labmedics, Stockport) at 37°C. Aggregation was measured as a change in turbidity (light transmission) in PRP or WP against a PPP or HEPES-tyrode reference respectively (Crane *et al*., 2002). Data were captured *via* an analogue digital converter (MacLab 4e, AD Instruments, Sussex, U.K.) and recorded using MacLab Chart v3.3.7. Aliquots (0.5 ml) of PRP or WP were equilibrated in the aggregometer at 37°C before the addition of DEA/NO (1 nM–10 μM), SNVP (10 nM–100 μM) or SIN-1 (3 nM–300 μM). Following incubation of drug for 1-min, supramaximal U46619 (a TxA_2 analogue; 8 μM) was added to the platelets to induce aggregation, and the response recorded for 5-min. In each case, the maximum response was measured and used for data analysis. U46619 was used in these experiments because it activates a signalling pathway downstream of platelet TxA_2 synthesis, minimising the effect of cGMP-independent inhibition of endogenous TxA_2 synthesis (Tsikas *et al*., 1999). In experiments designed to investigate cGMP-independent mechanisms, ODQ (20 μM) was preincubated with platelets for 15-min before the addition of DEA/NO, SNVP or SIN-1. In further experiments, aliquots of WP pretreated with ODQ were incubated with various plasma factors to determine the effect of releasing NO on cGMP-independent inhibition of platelet activation. In these experiments, CP (0.4 g l^{-1}) was added to WP for a 1-min period prior to SNVP (0.1–100 μM), which was added 1-min prior to U46619. Similarly, WP preincubated with ODQ were treated with supramaximal SOD (500 U ml^{-1}) for 1-min before the addition of SIN-1 (3 nM-300 μM), 1-min prior to U46619 ($n=6$ for all experiments). Control experiments examining the effect of incubation (15-min) of DMSO (0.1%) or ODQ (dissolved in DMSO; final concentration ODQ: 20 μM; DSMO: 0.1%) in both PRP and WP stimulated by U46619 (8 μM) were also performed ($n=4$).

Preparation of Fura-2 labelled WP

PRP was centrifuged (1200 × *g*, 10 min) in the presence of PGI_2 (300 ng ml^{-1}), and resuspended in 0.25 × HEPES-tyrode buffer. Fura-2 acetoxymethyl ester (Fura-2 AM; 2 μM) was added to the suspension and the mixture incubated at room temperature for 30 min. Following incubation, platelets were diluted to their original volume with HEPES-tyrode, PGI_2 added (300 ng ml^{-1}), and the mixture centrifuged (1200 × *g*, 10 min). The supernatant was aspirated and discarded to remove excess extracellular Fura-2, and the loaded platelets resuspended in HEPES-tyrode. Platelet count was then determined and standardized to $250\times10^9\,l^{-1}$ *via* dilution with HEPES-tyrode as described.

Ca^{2+} measurements

All fluorescence measurements were made using a Perkin-Elmer LS50B luminescence spectrometer (Perkin Elmer, Berkshire, U.K.). Fluorescence was measured at 37°C, with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Aliquots (1.5 ml) of Fura-2 loaded WP were equilibrated at 37°C before the addition of DEA/NO (10 μM), SIN-1 (100 μM), or SNVP (100 μM). After a 1-min incubation period, U46619 (8 μM) was added and the response measured for 5-min. These experiments were also repeated in platelets preincubated with ODQ (20 μM) for 15 min. Analogous to aggregometry experiments, aliquots of WP pretreated with ODQ were incubated with CP (0.4 g l^{-1}) for a 1-min period prior to the addition of SNVP (100 μM), which was added 1-min prior to U46619. Similarly, ODQ-treated WP preincubated with SOD (500 U ml^{-1}) for 1-min before the addition of SIN-1 (100 μM), 1-min prior to U46619. Ratio values were converted to intracellular Ca^{2+} concentration using FL WinLab software (Perkin Elmer, Berkshire, U.K.) with platelets solubilized in 1% Triton-X-100 followed by Ca^{2+} chelation with 20 mM EGTA to calculate the maximum and baseline values, respectively. The area under the curve (AUC) was calculated for all experiments ($n=4$–6).

Haemoglobin measurements

The haemoglobin content of PRP and WP was determined by colorimetric assay (Sigma Diagnostics, Dorset, U.K.) based on

the haemoglobin catalysed oxidation of 3,3′,5,5′-tetramethylbenzidine by hydrogen peroxide, with absorbance measured at 600 nm (Standefer & Vanderjagt, 1977; Lijana & Williams, 1979). Haemoglobin measurements were repeated 3–5 times.

Statistical analysis

All results are expressed as the mean ± s.e.m. unless otherwise stated. Concentration–response curves were analyzed by two-way analysis of variance (two-way ANOVA) where possible. Ca^{2+} data were analyzed *via* paired Student's *t*-test (DEA/NO) or one-way ANOVA followed by Dunnett's multiple comparison test (SNVP or SIN-1) of the calculated AUC. cGMP measurements and the DMSO/ODQ control aggregometry experiments were analyzed by one-way ANOVA. $P<0.05$ was considered to be statistically significant.

Results

cGMP measurements

Incubation of platelets with DEA/NO (10 μM) caused a significant ($P<0.01$) ~2-fold increase in platelet cGMP levels in both PRP and WP (in pmol/10^8 platelets: PRP control: 22.4 ± 4.9, + DEA/NO: 42.5 ± 4.3; WP control: 47.3 ± 3.0, + DEA/NO: 97.2 ± 9.8). Preincubation of platelets with ODQ at both concentrations tested (20 and 100 μM) completely prevented the DEA/NO-induced increase in cGMP in PRP and WP (in pmol/10^8 platelets: PRP: + 20 μM ODQ: 21.9 ± 2.7, + 100 μM ODQ: 22.3 ± 3.2; WP: + 20 μM ODQ: 43.2 ± 2.1, + 100 μM ODQ: 41.1 ± 2.9). In both cases, no significant difference to baseline cGMP levels was observed ($P>0.05$). Since 20 μM ODQ was sufficient to completely prevent DEA/NO-induced cGMP accumulation, this concentration was used in subsequent experiments to reduce nonspecific effects caused by the vehicle (DMSO).

Effect of DMSO and ODQ on platelet aggregation in PRP and WP

Incubation of DMSO (0.1%) or ODQ (20 μM) for 15-min in either PRP or WP did not significantly affect U46619-induced platelet aggregation (in mV: PRP control: 94.5 ± 7.6, + DMSO: 92.5 ± 8.8, + ODQ: 93.3 ± 7.8; WP control: 78.8 ± 8.4, + DMSO: 79.6 ± 9.4, + ODQ: 81.9 ± 9.5; $P>0.05$).

Generation of NO in PRP and WP by DEA/NO, SNVP and SIN-1

Addition of DEA/NO (3 μM), SNVP (100 μM) and SIN-1 (100 μM) at concentrations with maximal antiplatelet activity to PRP resulted in measurable NO generation from each compound (see Figure 1a). In all samples tested, there was a short lag phase (60–90 s) before NO was detected. In a parallel series of experiments, DEA/NO (3 μM), SNVP (100 μM) or SIN-1 (100 μM) was added to WP. High levels of NO were detected with DEA/NO (Figure 1b), while only low levels of NO were detected with SNVP (~40 nM; Figure 1b inset). NO was not detected with SIN-1, even under conditions of maximum electrode sensitivity (threshold ~10 nM; Figure 1b inset).

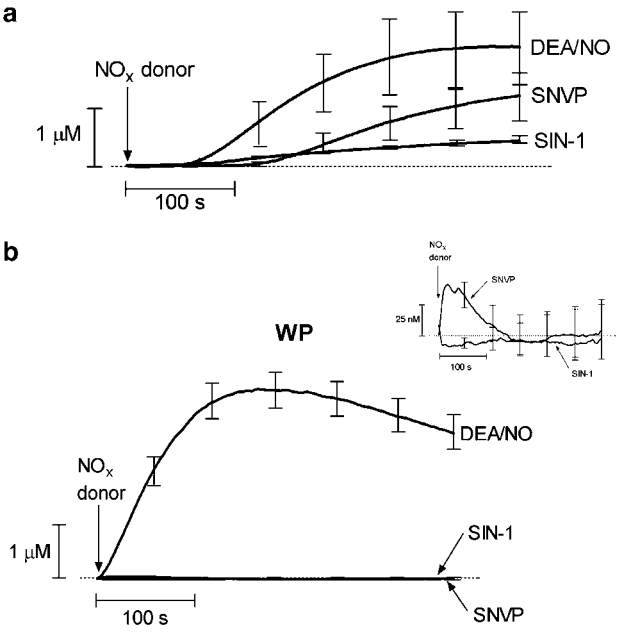

Figure 1 Generation of NO from DEA/NO, SNVP and SIN-1 in PRP and WP. Platelets were equilibrated at 37°C before the addition of DEA/NO (3 μM), SNVP (100 μM) or SIN-1 (100 μM) to PRP (a) or WP (b). Experiments involving the addition of SNVP (100 μM) and SIN-1 (100 μM) to WP are also shown on a smaller scale (inset). Data shown are the mean, with the s.e.m. indicated for every 60th (1-min) time point ($n=4$–5).

Inhibition of platelet aggregation in PRP and WP by DEA/NO

DEA/NO (1 nM–10 μM) inhibited U46619-induced platelet aggregation in PRP and WP in a concentration-dependent manner (Figure 2a(i–iii)). DEA/NO was approximately 100-fold more potent in WP compared to PRP. Preincubation of the sGC inhibitor (ODQ; 20 μM) with PRP for 15-min did not affect DEA/NO-mediated inhibition of platelet aggregation ($P>0.05$; control IC_{50}: 131 nM, + ODQ: 340 nM). However, in WP, ODQ inhibited DEA/NO-mediated inhibition of platelet aggregation, causing a right-shift of the concentration–response curve (control IC_{50}: 6.9 nM, + ODQ: 1.4 μM).

Inhibition of platelet aggregation in PRP and WP by SNVP

SNVP (10 nM–100 μM) also caused a concentration-dependent inhibition of U46619-induced platelet aggregation in PRP and WP (Figure 2b(i–ii)). Similarly to our observation with DEA/NO, SNVP (10 nM–100 μM) also inhibited platelet aggregation in WP at substantially lower concentrations than in PRP. Incubation of platelets with ODQ did not affect the inhibition of U46619-induced aggregation in PRP ($P>0.05$; control IC_{50}: 19.9 μM, + ODQ: 25.8 μM). However, ODQ abolished SNVP-mediated inhibition of aggregation in WP at all concentrations of SNVP tested (control IC_{50}: 270 nM).

Inhibition of platelet aggregation in PRP and WP by SIN-1

SIN-1 (3 nM–300 μM) also inhibited U46619-induced platelet aggregation in PRP and WP in a concentration-dependent

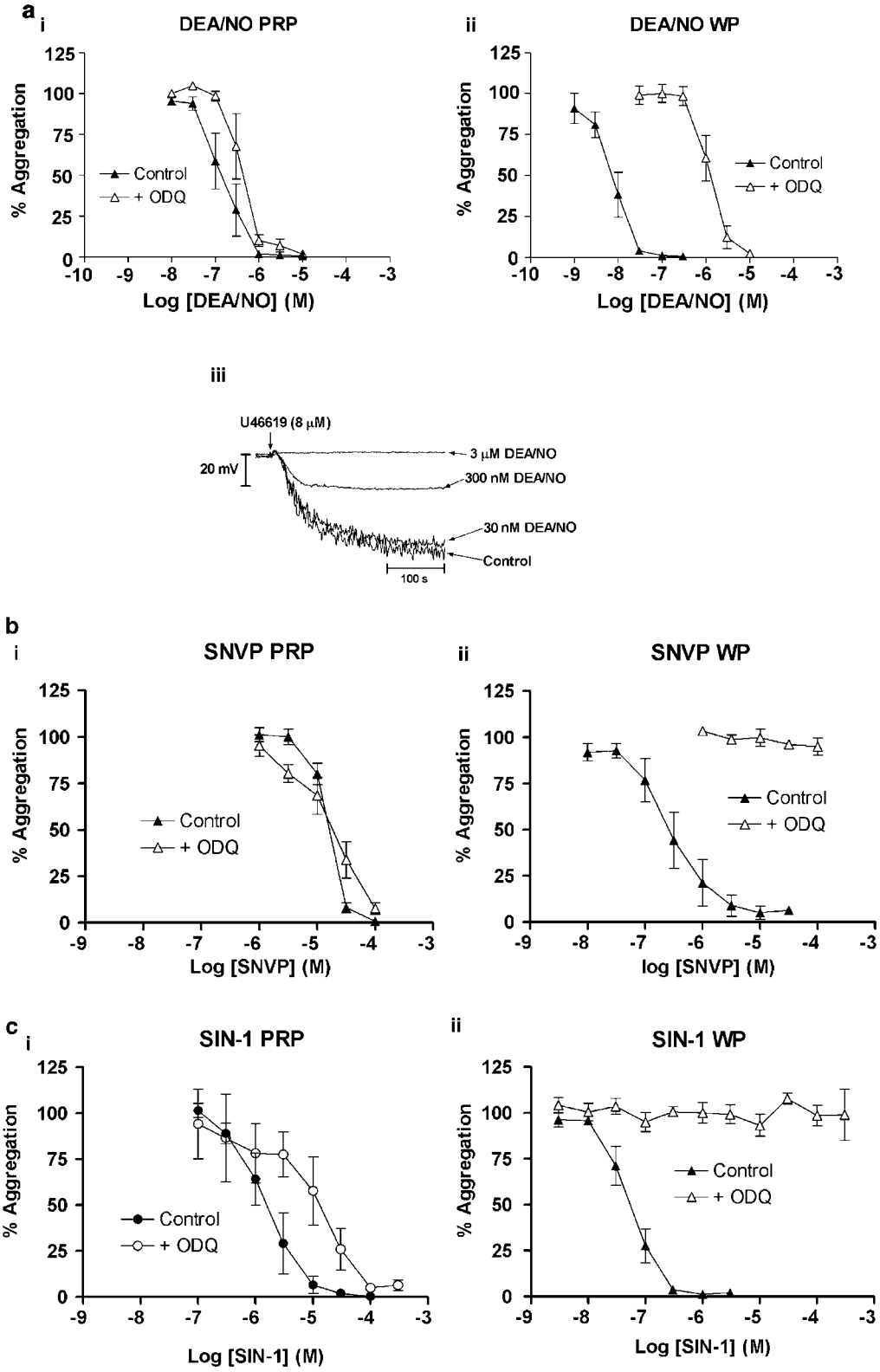

Figure 2 Inhibition of platelet aggregation by DEA/NO, SNVP and SIN-1 in PRP and WP in the presence and absence of ODQ. Platelets were equilibrated to 37°C before treatment with DEA/NO (a), SNVP (b) or SIN-1 (c) in PRP (i) or WP (ii). Platelet aggregation was then stimulated with U46619 (8 μM) 1-min after the addition of the NO_x donor. In experiments involving ODQ, platelets were preincubated with ODQ (20 μM) for 15-min before NO_x donor, followed by U46619 1-min later. ODQ did not affect DEA/NO and SNVP-mediated inhibition of aggregation in PRP ($P>0.05$), while it inhibited SIN-1-mediated inhibition of platelet aggregation in PRP ($P<0.05$). ODQ also inhibited DEA/NO-mediated inhibition of platelet aggregation in WP, and completely abolished SNVP and SIN-1-induced inhibition of platelet aggregation in WP. Data are expressed as the mean$\pm$s.e.m. ($n=6$). A representative trace of DEA/NO (30 nM–3 μM) in PRP (control) is included (iii).

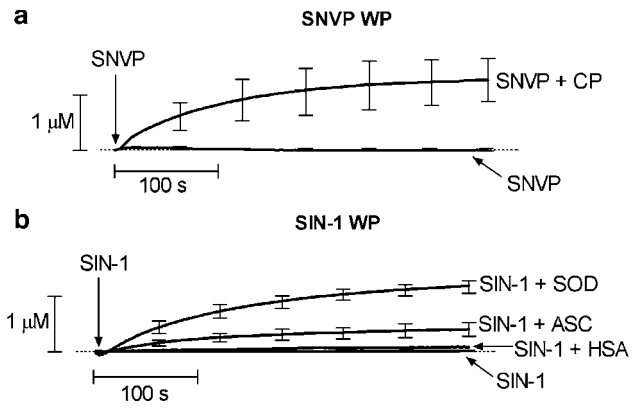

Figure 3 Effect of plasma factors on the generation of NO from SNVP and SIN-1 in WP. Platelets were equilibrated to 37°C before the addition of SNVP (100 μM; a) or SIN-1 (100 μM; b). In experiments involving SNVP, WP were preincubated with CP (0.4 g l^{-1}) for 1-min prior to the addition of SNVP. In SIN-1 experiments, WP were preincubated with SOD (500 U ml^{-1}), Asc (100 μM) or HSA (4%) for 1-min before the addition of SIN-1. Data shown are the mean, with the s.e.m. indicated for every 60th (1-min) time point ($n=4$).

manner (Figure 2c(i–ii)). SIN-1 was a considerably more potent inhibitor of platelet aggregation in WP compared to PRP. Incubation of ODQ caused a significant (~10-fold) rightward shift in the concentration–response curve for SIN-1 in PRP ($P<0.05$; control IC_{50}: 1.4 μM, +ODQ: 16.7 μM). In WP, ODQ abolished SIN-1 mediated inhibition of platelet aggregation at all concentrations tested (control IC_{50}: 54 nM).

Effect of plasma factors on the generation of NO by SNVP and SIN-1

In experiments designed to establish the effect of plasma factors on SNVP and SIN-1, WP was reconstituted with CP at a level that approximates its concentration in plasma (0.4 g l^{-1}; Ravin, 1961; Prakasam *et al*., 2001). CP was observed to greatly enhance the release of NO from SNVP (Figure 3a). Similarly, incubation of WP with SOD (500 U ml^{-1}) prior to the addition of SIN-1 resulted in measurable NO generation (Figure 3b). Furthermore, incubation of WP with Asc (100 μM) and HSA (4%) prior to SIN-1 also resulted in measurable NO generation from SIN-1 (Figure 3b).

Effect of CP on SNVP-mediated inhibition of platelet aggregation in WP

Reconstitution of WP with CP (0.4 g l^{-1}) in the presence of ODQ prior to treatment with SNVP resulted in concentration-dependent inhibition of platelet aggregation (Figure 4a; IC_{50}: 3.3 μM).

Effect of SOD on SIN-1 mediated inhibition of platelet aggregation in WP

In a parallel experiment, reconstitution of WP with SOD (500 U ml^{-1}) in the presence of ODQ before treatment with SIN-1 also resulted in concentration-dependent inhibition of aggregation (Figure 4b; IC_{50}: 30.0 μM).

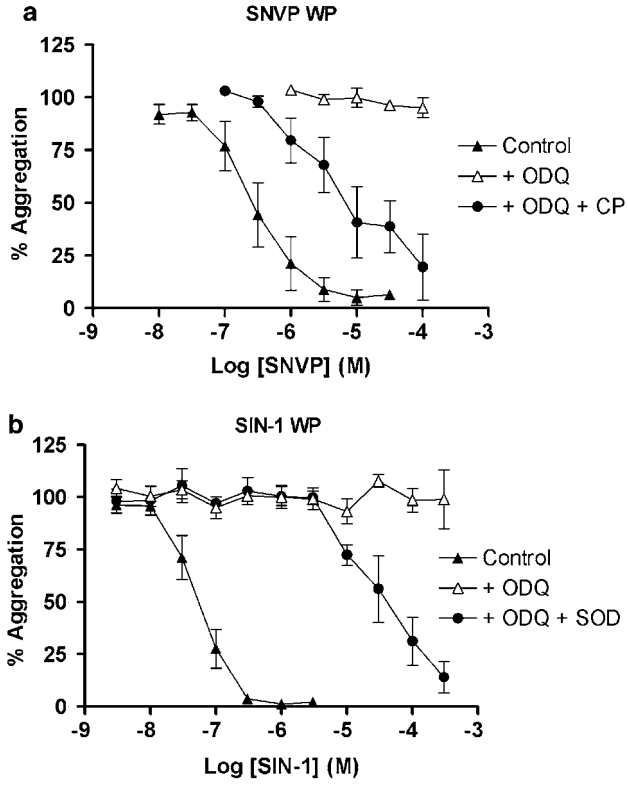

Figure 4 Effect of plasma factors on cGMP-independent inhibition of platelet aggregation by SNVP and SIN-1 in WP. Platelets equilibrated to 37°C were preincubated with ODQ (20 μM) for 14-min before the addition of CP (0.4 g l^{-1}) 1-min prior to the addition of SNVP (a). SOD (500 U ml^{-1}) was also added to ODQ-treated WP 1-min before the addition of SIN-1 (b). After incubation with SNVP or SIN-1 for 1-min, U46619 (8 μM) was added to induce aggregation. Previous data are added as a comparison. Data are expressed as the mean ± s.e.m. ($n=6$).

Effect of NO_x donors on Ca^{2+} signalling in Fura-2 loaded WP

Addition of U46619 to fura-2 loaded WP caused an expected rapid Ca^{2+} spike, followed by a sustained elevation of intracellular Ca^{2+} levels. Preincubation of WP with DEA/NO (10 μM) inhibited this Ca^{2+} signalling, an effect that was not blocked by ODQ (Figure 5a(i); $P>0.05$). Incubation of WP with SNVP (100 μM) also inhibited U46619-induced Ca^{2+} signalling, but this effect was blocked by ODQ (Figure 5a(ii), $P<0.001$). Preincubation of ODQ-treated WP with CP 1-min prior to SNVP reversed the antagonistic effect of ODQ on SNVP ($P<0.001$), to a similar level observed with the *S*-nitrosothiol alone (Figure 5a(ii)). Similarly, incubation of WP with SIN-1 (100 μM) resulted in an inhibitory effect on U46619-induced Ca^{2+} signalling that was reversed by ODQ (Figure 5a(iii)). Preincubation of ODQ-treated WP with SOD also prevented the inhibitory action of ODQ on SIN-1 ($P<0.001$, Figure 5a(iii)). Summary data showing this trend are also presented (Figure 5b).

Effect of ODQ on NO generation by the NO_x donors in PRP

In experiments investigating the effect of ODQ (20 μM) on NO generation by the NO_x donors in PRP, ODQ was observed to

substantially reduce the length of the lag phase observed with all three donor drugs, without altering the maximum concentration of NO detected (Figure 6a–c).

Haemoglobin measurements

The haemoglobin concentration in PRP was $0.35 \pm 0.03\,\mu$M, while haemoglobin was undetectable in WP.

Discussion

These results suggest that NO-mediated cGMP-independent antiplatelet effects are reliant on the generation of NO (radical) in the extracellular compartment. DEA/NO inhibited platelet aggregation *via* cGMP-independent mechanisms in both PRP and WP, implying a role for exogenous NO in cGMP-independent inhibition of activation. SNVP, however, inhibited platelet aggregation *via* a cGMP-independent mechanism in PRP, but inhibition in WP was entirely dependent on cGMP. Analysis of NO generation by a high concentration of SNVP ($100\,\mu$M) using an isolated electrode revealed that SNVP generated significant amounts ($\sim 1\,\mu$M) of NO in PRP sustained throughout a 5-min period, but in WP only generated a small transient increase in extracellular NO (~ 40 nM) that persisted for only $\sim$2-min. Reconstitution of WP with the copper-containing protein, CP, at similar levels to those found in plasma elicited extracellular release of NO and conferred cGMP-independent inhibition of platelet aggregation to SNVP. Incubation of platelets with SIN-1 revealed a similar trend to that observed with SNVP, with cGMP-independent inhibition of aggregation only observed in PRP and not WP. SIN-1 only generated detectable NO in PRP, but incubation of WP with SOD resulted in detectable generation of extracellular NO from SIN-1, and also caused cGMP-independent inhibition of aggregation. Experiments with fura-2 loaded platelets demonstrated that both CP and SOD conferred cGMP-independent inhibition of Ca^{2+} signalling by SNVP and SIN-1, respectively, indicating that the cGMP-independent target(s) play a role in the regulation of platelet Ca^{2+} signalling. Taken together, these data suggest a requirement for exogenous NO in the generation of cGMP-independent inhibition of platelet activation through inhibitory effects on Ca^{2+} mobilization.

Confidence in the inhibitory effects of ODQ on sGC is essential to facilitate interpretation of the data derived from this study. Initial experiments were performed to assess the concentration of ODQ required to prevent cGMP formation to a maximal concentration of DEA/NO ($10\,\mu$M). ODQ has previously been used at a concentration of $20\,\mu$M (or less) to

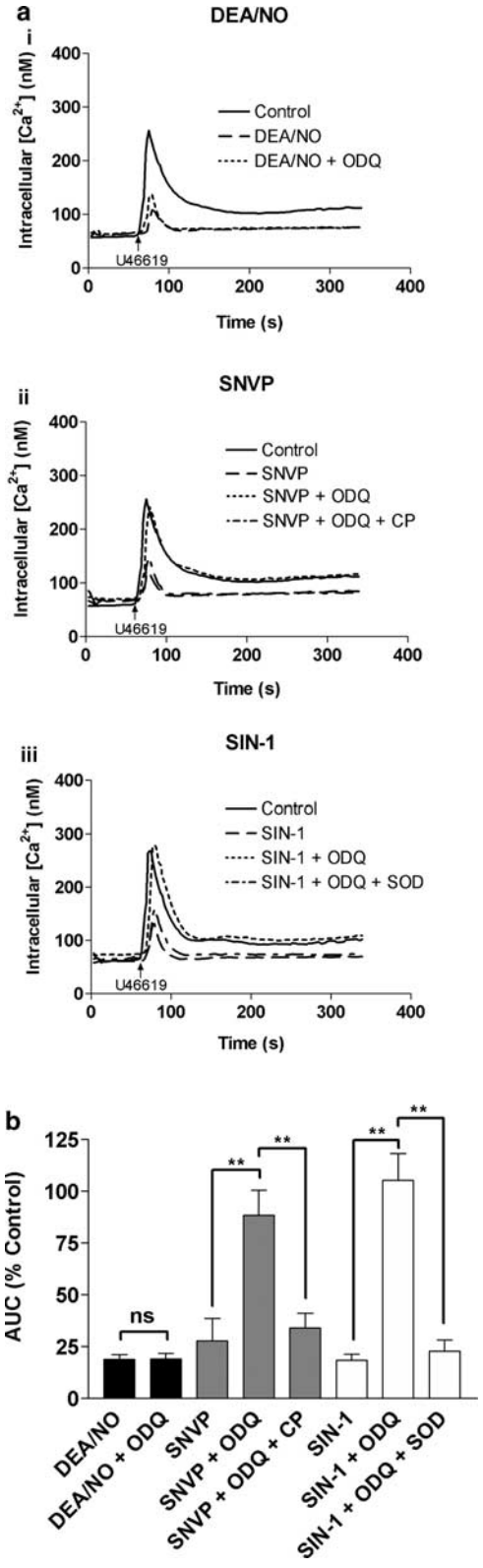

Figure 5 Effect of DEA/NO, SNVP and SIN-1 on Ca^{2+} signalling in Fura-2 labelled WP. Platelets loaded with Fura-2 were equilibrated to 37°C before the addition of DEA/NO ($10\,\mu$M – a(i)), SNVP ($100\,\mu$M – a(ii)) or SIN-1 ($100\,\mu$M – a(iii)). In experiments involving ODQ, WP were preincubated with ODQ ($20\,\mu$M) for 15-min before the addition of NO_x donor. In other experiments, ODQ-treated WP were reconstituted with CP ($0.4\,g\,l^{-1}$) or SOD ($500\,U\,ml^{-1}$) before the addition of SNVP or SIN-1 respectively. Representative traces are included (i–iii) alongside summary data obtained by measuring the AUC (b). ODQ did not affect DEA/NO-mediated inhibition of Ca^{2+} signalling ($P > 0.05$); however, it significantly attenuated the inhibitory action of SNVP and SIN-1 on Ca^{2+} signalling ($P < 0.001$). The effect of ODQ was significantly reversed by the addition of CP and SOD to SNVP and SIN-1, respectively ($P < 0.001$). Summary data are expressed as the mean $\pm$ s.e.m. ($n = 4$–6).

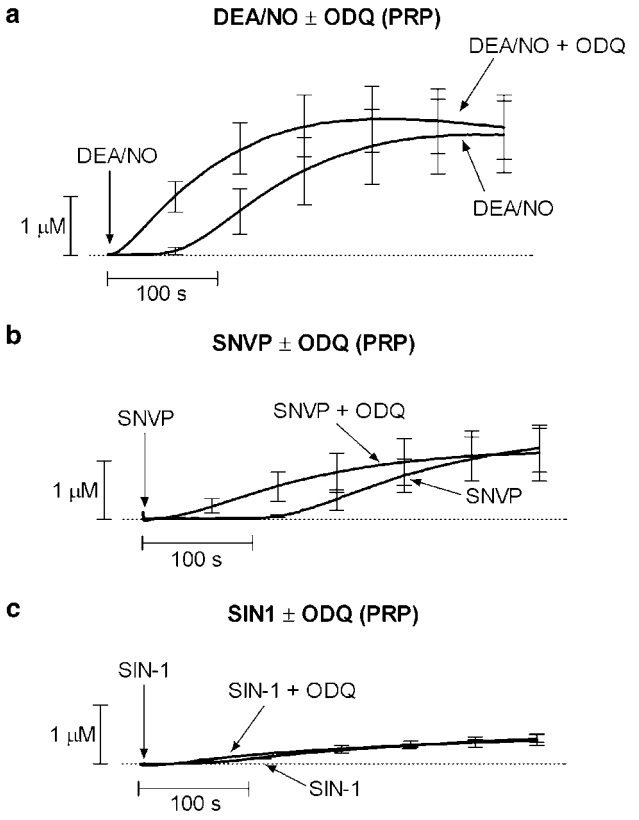

Figure 6 Effect of ODQ incubation on NO generation by DEA/NO, SNVP and SIN-1 in PRP. Platelets were equilibrated to 37°C before the addition of DEA/NO (3 μM – a), SNVP (100 μM – b) or SIN-1 (100 μM – c). In experiments involving ODQ, PRP was treated with ODQ (20 μM) for 15-min before the addition of DEA/NO, SNVP or SIN-1. Data shown are the mean, with the s.e.m. indicated for every 60th (1-min) time point ($n=4$).

investigate NO-mediated cGMP-independent antiplatelet effects (Tsikas *et al*., 1999; Homer & Wanstall, 2002); however, a 100 : 1 excess of the NO donor SNAP can result in a partial reversal of ODQ-mediated inhibition of sGC (Moro *et al*., 1996). In our experiments, a theoretical maximum of 20 μM NO will be released by 10 μM DEA/NO, which is equivalent to the concentration of ODQ used here, and unlikely to be sufficiently high to overcome ODQ-mediated inhibition. cGMP measurements revealed that 20 μM ODQ was sufficient to completely prevent NO-mediated cGMP formation and that no added benefit was observed when the ODQ concentration was increased to 100 μM. Furthermore, the observation that 20 μM ODQ was sufficient to completely prevent SNVP and SIN-1-mediated inhibition of aggregation in WP at concentrations 1000-fold greater than that required to inhibit aggregation confirms the notion that these NO-donors do not release sufficient NO intracellularly to overcome ODQ-mediated inhibition of sGC. Importantly, neither ODQ nor its vehicle (DMSO) affected U46619-induced platelet aggregation in PRP or WP. These experiments therefore support the hypothesis that the inhibition of aggregation observed in the presence of 20 μM ODQ represents genuine cGMP-independent responses.

DEA/NO hydrolyses in physiological solutions with a half-life of ~2 min at physiological temperature and pH (Davies *et al*., 2001). Importantly, biological factors are not required to drive DEA/NO hydrolysis; therefore, DEA/NO will generate equivalent amounts of NO in both PRP and buffer. SNVP, a more stable and lipophilic analogue of the well-recognized S-nitrosothiol, SNAP, was chosen for these studies because it is relatively stable but nevertheless undergoes transnitrosation reactions (Megson *et al*., 1999): it is a useful tool in establishing a role for transnitrosation in cGMP-independent effects. SIN-1 was originally believed to be a NO donor, but is now known to generate O_2^- concurrently with NO (Feelisch *et al*., 1989; Noack & Feelisch, 1991; Taylor *et al*., 2004). The reaction rate between NO and O_2^- is near diffusion limited (rate constant $= 6.7 \times 10^9\,M^{-1}\,s^{-1}$; Huie & Padmaja 1993), making SIN-1 an effective and convenient $ONOO^-$ donor.

Our results indicate that in WP, all three NO_x donors stimulate sGC, inhibit Ca^{2+} signalling and prevent platelet aggregation. The EC_{50} for DEA/NO in WP was ~10 nM (Figure 2a(ii)), indicating that low nM concentrations of NO ($\leqslant$20 nM) are sufficient to stimulate sGC. A key finding in experiments here is that cGMP-independent antiplatelet effects are only observed in conditions where extracellular NO is detectable. Thus, while all NO_x donors generated extracellular NO in PRP and induced cGMP-independent inhibition of aggregation, only DEA/NO generated substantial and sustained levels of extracellular NO and induced cGMP-independent antiplatelet effects in WP (EC_{50} $\leqslant$2 μM NO; Figures 1 and 2). At this juncture, it is therefore unclear whether the absolute NO concentration or the site of its release is the important factor for cGMP-independent antiplatelet effects. This supposition may be answered most clearly *via* analysis of the data obtained for SIN-1. In WP, SIN-1 exhibited only cGMP-dependent inhibition of platelet aggregation (EC_{50} ~50 nM; Figure 2c(ii)) and even at high concentrations (100 μM) did not generate detectable levels of extracellular NO (limit of detection ~10 nM; Figure 1b). Thus, 50 nM SIN-1 is sufficient to generate the low nM amounts of intracellular NO required to stimulate sGC. Although we can only estimate intracellular NO levels, assuming that the addition of maximal concentrations of SIN-1 (300 μM) to WP results in a similar-fold increase in intracellular NO (i.e. a 6000-fold increase of intracellular NO to levels in the μM range), the manifestation of cGMP-independent effects would be expected if the concentration of NO is the determining factor. However, cGMP-independent effects with SIN-1 are only observed when extracellular NO is detected (i.e. in PRP or when NO is unmasked by the membrane-impermeant protein SOD). Indeed, the EC_{50} for SIN-1 in the presence of SOD is ~30 μM (Figure 4b), implying that cGMP-independent effects may occur at lower levels (i.e. in the nM range) than those predicted by the DEA/NO concentration–response curve. These data therefore support the hypothesis that the site of NO generation is an important factor governing cGMP-independent antiplatelet effects. This premise is further supported by the SNVP data. Although the addition of SNVP to WP resulted in a small, transient increase in extracellular NO (~40 nM from 100 μM SNVP; Figure 1b), this was insufficient to elicit cGMP-independent effects, suggesting that concentrations of extracellular NO exceeding 30 nM are required for cGMP-independent antiplatelet effects. The addition of the plasma protein CP induced the extracellular release of NO and stimulated cGMP-independent inhibition of platelet aggregation. Interestingly, data indicate that 100 μM

SNVP in the presence of CP results in the generation of $\sim 1\,\mu$M NO (Figure 3a), yet the EC_{50} for cGMP-independent inhibition of aggregation for SNVP in the presence of CP was $\sim 10\,\mu$M (Figure 4a). This therefore supports the premise that cGMP-independent effects may occur at levels of extracellular NO in the nM range.

These results indicate that NO inhibits Ca^{2+} signalling *via* a cGMP-independent mechanism. To date, numerous potential targets for cGMP-independent inhibition have been established. There is convincing evidence that NO can activate the platelet sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA; Trepakova *et al*., 1999; Homer & Wanstall, 2002). Our results suggest that the cGMP-independent effects of NO do indeed impact on Ca^{2+} trafficking (Figure 5), in agreement with earlier findings. However, the apparent role for extracellular NO in cGMP-independent inhibition of platelet activation might suggest that NO-mediated modification of cell surface components is a more likely target than an intracellular component that has to compete for NO with high affinity sGC found throughout the cytoplasm. This discrepancy may be explained by the observation that SERCA is located in the dense tubular system (DTS; Horiguchi *et al*., 1998), in close proximity to the open cannalicular system and plasma membrane. Therefore, in platelets, SERCA may be in an ideal position proximal to the outer surface of the platelet to detect NO generated in the extracellular environment, and respond to increases in NO by enhancing sequestration of Ca^{2+} back within the DTS. However, other cGMP-independent mechanisms including the interaction of NO with platelet surface thiols may also play a role as previously implied (Gordge *et al*., 1998; Sogo *et al*., 2000).

The physiological implications of data presented here is unclear. Concentration–response curves in PRP$\pm$ODQ are difficult to compare on a quantitative level on account of the fact that ODQ will oxidize residual haemoglobin present in PRP (Figure 6; Zhao *et al*., 2000), reducing its ability to bind NO and thereby effectively increasing the NO dose received by these platelets. Indeed, the removal of haemoglobin in the washing procedure is likely to explain the increased NO-sensitivity observed in WP compared to PRP. However, cell-free Hb is present within blood *in vivo* (Lentener, 1984) and represents a significant barrier for NO-mediated platelet effects, irrespective of the NO source. The fact that physiological concentrations of plasma constituents such as CP and Asc or HSA accelerate the release of NO from SNVP and SIN-1 respectively (Figure 3) and that haemoglobin-mediated scavenging of NO will have to be overcome before any antiplatelet effects are observed will mean that cGMP-independent effects of these drugs are likely to be evoked.

In summary, our data suggest a requirement for the extracellular generation of NO (>40 nM) to stimulate cGMP-independent inhibition of platelet activation. Plasma antioxidants and proteins such as CP can evoke cGMP-independent antiplatelet activities on *S*-nitrosothiols and $ONOO^-$ generators by accelerating the release of NO from these compounds, suppressing platelet Ca^{2+} signalling events and inhibiting platelet function.

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