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Sildenafil potentiates nitric oxide mediated inhibition of human platelet aggregation

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Abstract

Nitric oxide (NO) inhibits platelet aggregation primarily via a cyclic 3′5′-guanosine monophosphate (cGMP)-dependent process. Sildenafil is a phosphodiesterase type 5 (PDE5) inhibitor that potentiates NO action by reducing cGMP breakdown. We hypothesised that sildenafil would augment the inhibitory effects of NO on in vitro platelet aggregation. After incubation with sildenafil or the soluble guanylate cyclase inhibitor *H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxallin-1-one (ODQ), collagen-mediated human platelet aggregation was assessed in the presence of two NO donors, the cGMP-dependent sodium nitroprusside (SNP) and the cGMP-independent diethylamine diazeniumdiolate (DEA/NO). SNP and DEA/NO caused a concentration-dependent inhibition of platelet aggregation. ODQ inhibited and sildenafil augmented the effect of SNP, and to a lesser extent the effect of DEA/NO. We conclude that sildenafil potentiates NO-mediated inhibition of platelet aggregation through blockade of cGMP metabolism and that PDE5 inhibitors may have important antiplatelet actions relevant to the prevention of cardiovascular disease.

Keywords: Nitric oxide; cGMP; Phosphodiesterase inhibitors; Platelet aggregation

Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet aggregation. It is generated constitutively by nitric oxide synthase (NOS) in endothelial cells and platelets, or it can be derived from an exogenous source such as NO donor drugs. The effects of NO are predominantly mediated via stimulation of soluble guanylate cyclase (sGC) which catalyses synthesis of the secondary messenger cyclic 3′5′-guanosine monophosphate (cGMP) from guanosine 5′-triphosphate (GTP) [1]. cGMP reduces platelet adhesion and aggregation via predominantly protein kinase C (PKC)-mediated pathways [2].

Phosphodiesterases are a group of enzymes responsible for the breakdown of cyclic nucleotides including cyclic 3′5′-adenosine monophosphate (cAMP) and cGMP. Phosphodiesterase type 5 (PDE5) is highly specific for cGMP metabolism, with inhibition of PDE5 leading to accumulation of cGMP and augmentation of the effects of NO. Sildenafil is a selective inhibitor of PDE5 and was originally developed as a potential antianginal drug because it augments the vasodilator effects of both endogenous NO, such as from the endothelium or platelets, and exogenous NO, such as from NO donor drugs. Although an ineffective antianginal therapy, it is an efficacious treatment for erectile dysfunction by augmenting the physical response to sexual stimulation and increasing penile blood flow by relaxing smooth muscle in the corpus cavernosum [3,4]. Recently, there has been renewed interest in the therapeutic potential of PDE5 inhibitors for other cardiovascular indications and small trials have shown benefit in the treatment of both primary [5–10] and secondary [11] pulmonary hypertension.

Given the importance of platelet activation in thrombosis associated with cardiovascular disease, it is important to

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determine whether sildenafil also affects platelet aggregation. Antiplatelet effects of sildenafil would have therapeutic implications for patients with cardiovascular disease both with respect to its potential use as a primary treatment and because patients requiring sildenafil for impotence frequently have other cardiovascular conditions. Previously published studies on the effects of sildenafil on platelet function have been inconclusive and contradictory [3,12,13].

We hypothesised that sildenafil would augment the inhibitory effects of NO donor drugs on collagen mediated human platelet aggregation in vitro and that the effects of sildenafil would be affected by the relative cGMP dependence of the NO donors. We therefore compared the effects of sildenafil on two different NO donors, sodium nitroprusside (SNP) and diethylamine diazeniumdiolate (DEA/NO), that inhibit platelet aggregation through cGMP-dependent and -independent mechanisms, respectively.

Materials and methods

Materials. Sildenafil (Pfizer, Sandwich, UK) was diluted in saline and kept at room temperature. ODQ (Tocris Cookson, Langford, UK) was dissolved in dimethyl sulphoxide (DMSO) prior to dilution in saline; final concentrations of DMSO did not exceed 0.5%. DEA/NO (Alexis, Nottingham, UK) was dissolved in 0.01 M NaOH. SNP (Sigma chemicals, USA) was dissolved in saline and protected from light. Collagen was obtained from Chrono-Log, Havertown, USA. All other reagents were obtained from Sigma, Poole, Dorset, UK.

Samples. Peripheral venous blood was drawn from healthy human non-smokers ($n = 25$) who had not taken any medications including anti-inflammatory drugs for at least 10 days. Blood was collected from the ante-cubital fossa using a 19 gauge butterfly needle into a 50 mL syringe and transferred into tubes containing 3.8% sodium citrate. The blood was centrifuged at 130*g* for 20 min at room temperature to obtain platelet rich plasma (PRP) and the platelet count was adjusted to levels between 200 and 250×10^9/L using autologous platelet poor plasma (PPP). Blood was centrifuged at 1200*g* for 10 min to obtain PPP for this purpose and for reference samples.

Platelet aggregation. Platelet aggregation was measured using standard optical platelet aggregometry [14]. PRP samples were equilibrated at 37 °C and stirred continuously. Platelets were incubated with sildenafil, 1 μM [3,12], for 5 min, or the selective sGC inhibitor ODQ, 20 μM [15], for 15 min. PRP was then treated with the NO-donors SNP or DEA/NO (0.1–10 μM; 1 min), before induction of platelet aggregation with a supramaximal concentration of collagen (2.5 μg/mL). Aggregation was monitored for 5 min in a four channel aggregometer (Chronolog 470 VS, Labmedics, Stockport, UK) linked to a MacLab 4s analogue-digital converter (AD Instruments, Sussex, UK) and Chart software (AD instruments, Sussex, UK). All results are expressed as a percentage of the maximal collagen-induced aggregation.

Statistical analysis. Data were analysed using GraphPad Prism software and results are expressed as means ± SEM. Statistical significance was taken at 5% level. Two-way analysis of variance (ANOVA) was used to assess differences between the groups.

Results

Addition of both NO-donor drugs, SNP and DEA/NO, at concentrations of 0.1–10 μM caused concentration-dependent inhibition of collagen (2.5 μg/mL) mediated platelet aggregation ($n = 25$; ANOVA, $p < 0.0001$ for both SNP and DEA/NO; Fig. 1).

cGMP-dependence of SNP and DEA/NO

The inhibitory effects of SNP (0.1–10 μM) on collagen mediated platelet aggregation were almost abolished by 15 min incubation with ODQ (20 μM) ($n = 11$; ANOVA, $p < 0.001$; Fig. 1A). ODQ also reduced the inhibitory effects of DEA/NO ($n = 12$, $IC_{50} = 4.7 \times 10^{-9}$ M versus 8.2×10^{-7} M, $p < 0.001$; Fig. 1B) but to an apparently lesser degree than those of SNP.

Effects of sildenafil on NO mediated inhibition of platelet aggregation

Pre-incubation with sildenafil (1 μM) for 5 min augmented the inhibitory effects of both SNP ($IC_{50} = 2.6 \times 10^{-6}$ M versus 3.6×10^{-7} M; $n = 14$; ANOVA, $p < 0.0001$; Fig. 2A) and to a lesser extent than those of DEA/NO ($IC_{50} = 1.6 \times 10^{-7}$ M versus 1×10^{-7} M; $n = 13$; ANOVA, $p < 0.0001$; Fig. 2B). Sildenafil (1 μM) had no effects on its own (62 ± 3% versus 62 ± 4%; $n = 17$, $p =$ ns, paired *t* test) on collagen mediated platelet aggregation.

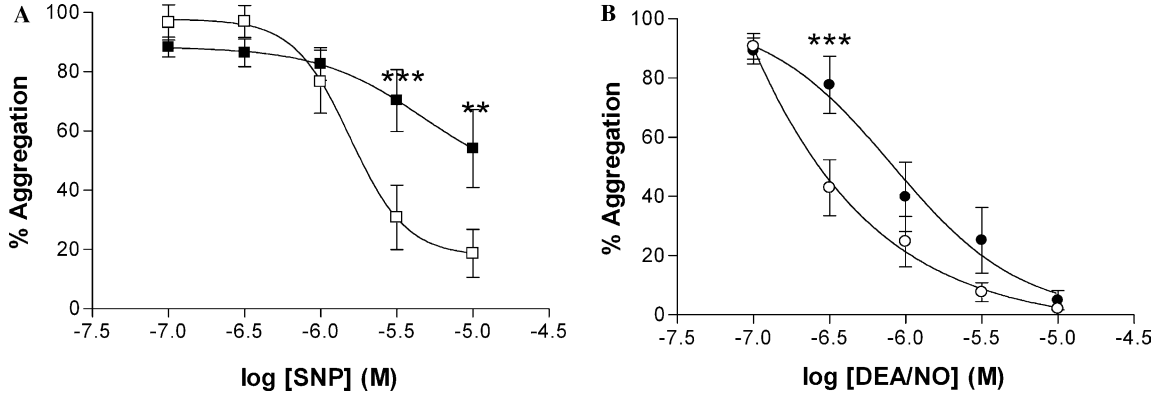

Fig. 1. (A,B) Inhibition of collagen induced platelet aggregation by sodium nitroprusside (SNP; squares) and diethylamine diazeniumdiolate (DEA/NO; circles) in the presence (closed symbols) or absence (open symbols) of *H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxallin-1-one (ODQ). $^{***}p < 0.001$, $^{**}p < 0.01$.

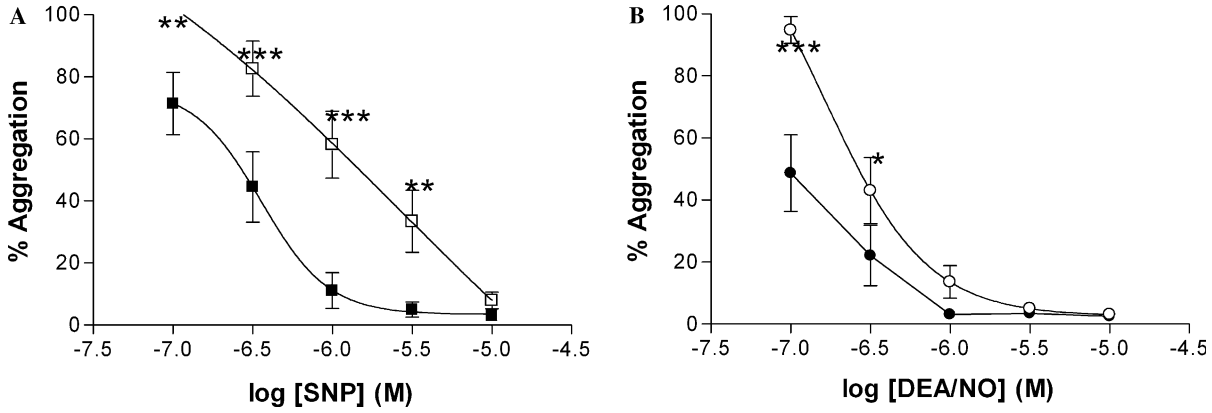

Fig. 2. (A,B) Inhibition of collagen induced platelet aggregation by sodium nitroprusside (SNP; squares) and diethylamine diazeniumdiolate (DEA/NO; circles) in the presence (closed symbols) or absence (open symbols) of sildenafil. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

Discussion

We have shown that sildenafil alone does not inhibit platelet aggregation but has a powerful potentiating effect on the antiplatelet effects of NO donor drugs. In keeping with the relative cGMP-dependence of the two NO donor drugs tested, SNP appeared to be more sensitive to sildenafil-mediated augmentation. We conclude that sildenafil predominantly potentiates cGMP-dependent NO mediated inhibition of platelet aggregation. This may have relevance to the potential cardiovascular effects of sildenafil.

We selected two different NO donors for our studies, SNP and DEA/NO. SNP is a cGMP-dependent NO donor that acts by diffusing into the platelet where it undergoes bioactivation to release NO and activate sGC, leading to the production of cGMP. In contrast, DEA/NO is hydrolysed spontaneously at physiological temperature and pH, and releases NO primarily in the extracellular compartment, resulting in antiplatelet effects that are largely cGMP-independent [14,15]. Separate experiments with similar diazeniumdiolates indicate that these effects are mediated by increased sequestration of Ca^{2+} into the sarcoplasmic reticulum through activation of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) [16]. In keeping with previous studies [15], the sGC inhibitor ODQ nearly completely inhibited the effects of SNP, suggesting cGMP dependence, whilst it only modestly affected DEA/NO. Furthermore, sildenafil was found to potentiate the effects of both SNP and DEA/NO, but this effect was most marked with SNP.

Previously published studies have been inconclusive and inconsistent. Some have suggested that sildenafil might augment thrombin induced platelet aggregation [12], whilst others describe an independent inhibitory effect of sildenafil [17]. Consistent with our own findings, Wallis et al. [3] reported that sildenafil does not have direct effects, but does augment the inhibitory effects of an NO donor. The lack of effect of sildenafil alone on collagen-induced platelet aggregation is perhaps surprising, given that platelets are known to contain constitutive nitric oxide synthase and to generate sufficient NO to modulate aggregatory responses [18,19]. It is, however, worth noting that activation of constitutive nitric oxide synthases is highly dependent on intracellular Ca^{2+} that, in the case of platelets, is only elevated during activation. Faced with a strong stimulus in the form of collagen, it is likely that any increase in cGMP levels will be "too little, too late" to have any impact on the aggregation process. It does not, however, rule out an in vivo effect of sildenafil on the activation process. Platelets are continuously exposed to endothelium-derived NO as well as that from platelets themselves, and at least some of the activation stimuli are less intensive than the in vitro exposure to collagen.

We conclude that PDE 5 inhibitors may have important antiplatelet effects that have potential in the treatment of cardiovascular diseases.

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