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RESEARCH PAPER

The anti-aggregating effect of BAY 41-2272, a stimulator of soluble guanylyl cyclase, requires the presence of nitric oxide

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BACKGROUND AND PURPOSE
The purpose of the present study was to determine whether a stimulator of soluble guanylyl cyclase, BAY 41-2272, inhibits platelet aggregation and to clarify its interaction with nitric oxide (NO).

EXPERIMENTAL APPROACH
Blood was collected from anaesthetized Wistar Kyoto rats. The aggregation of washed platelets was measured and the production of cAMP and cGMP was determined.

KEY RESULTS
In adenosine 5′-diphosphate (ADP)-induced platelet aggregation, the anti-aggregating effects of BAY 41-2272, nitroglycerin, sodium nitroprusside and DEA-NONOate were associated with increased levels of cGMP while that of beraprost, a prostacyclin analogue, was correlated with an increase in cAMP. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) prevented the effects of BAY 41-2272 and that of nitroglycerin and sodium nitroprusside, but only inhibited the increase in cGMP produced by of DEA-NONOate. Hydroxocobalamin, an NO scavenger, inhibited the effects of the three NO donors and BAY 41-2272 but did not affect those of beraprost. ADP-induced aggregation and the effects of BAY 41-2272 were not affected by L-nitroarginine. A positive interaction was observed between BAY 41-2272 and the three NO donors. BAY 41-2272 potentiated also the anti-aggregating effects of beraprost, and again this potentiation was inhibited by hydroxocobalamin.

CONCLUSIONS AND IMPLICATIONS
Inhibition of platelet aggregation by BAY 41-2272 requires the reduced form of soluble guanylyl cyclase and the presence of NO. The positive interaction observed between BAY 41-2272 and various NO donors is qualitatively similar whatever the mechanism involved in NO release. Furthermore, a potent synergism is observed between BAY 41-2272 and a prostacyclin analogue, but only in the presence of NO.

Abbreviations
BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; BAY 58-2667, 4-[((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino) methyl [benzoic]acid; DEA-NONOate, 2-(N,N-diethylamino)-diazenolate 2-oxide sodium salt hydrate; DMSO, dimethyl sulphoxide; IBMX, isobutyl-methyl-xanthine; L-NA, L-nitro-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; WKY, Wistar Kyoto; YC-1, 3-(5′hydroxymethyl-2′-furyl)-1-benzyl indazole

Introduction

Nitric oxide (NO), originally identified as a powerful vasodilator, also plays important biological roles in the gastrointestinal, respiratory, nervous and immune systems. Additionally, it is a potent anti-thrombotic agent (Furchgott and Zawadzki, 1980; Furchgott, 1988; Ignarro *et al.*, 1988; Moncada *et al.*, 1991). Most of the physiological effects of NO, including the inhibition of platelet aggregation, are

associated with the activation of soluble guanylyl cyclase (Rapoport and Murad, 1983; Radomski *et al.*, 1987; Bhardwaj *et al.*, 1988; Alexander *et al.*, 2009).

Soluble guanylyl cyclase is a heterodimer, consisting of an α- and a β-subunit, the latter containing the prosthetic ferrous haem group (Fe^{2+}) that catalyses the conversion of GTP to the second messenger cGMP (Ignarro, 1991). NO binds directly to the haem group and cannot activate the enzyme when it is haem-free (Lucas *et al.*, 2000) or when the haem moiety is oxidized (Fe^{3+}). The latter occurs following oxidative stress or under the action of inhibitors such as 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Garthwaite *et al.*, 1995; Stasch *et al.*, 2006). Pharmacological agents have been synthesized that interact with the soluble guanylyl cyclase and generate cGMP. Based on their mechanisms of action, these compounds can be separated into two different classes, the stimulators and the activators of soluble guanylyl cyclase (Schmidt *et al.*, 2009; Stasch and Hobbs, 2009). The former class of compounds, which includes BAY 41-2272 [(5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)], stimulates the enzyme in an NO-independent but haem-dependent manner and, as allosteric modulators, markedly enhance NO-dependent cGMP production (Stasch *et al.*, 2001; Evgenov *et al.*, 2006).

BAY 41-2272 is a potent vasodilator and inhibitor of platelet aggregation (Stasch *et al.*, 2001; Hobbs and Moncada, 2003). Both NO and prostacyclin exhibit synergistic activity with BAY 41-2272 to inhibit platelet activity (Hobbs and Moncada, 2003). However, NO gas and NO donor drugs, especially those that generate NO spontaneously, can exert cGMP-independent vasorelaxation and inhibition of platelet aggregation (Wanstall *et al.*, 2005). Likewise, besides stimulating the soluble guanylyl cyclase, BAY 41-2272 may also inhibit, in a cGMP-independent manner, calcium entry, $Na^+/K^{\pm}$ATPase and, at elevated concentrations, phosphodiesterase type 5 (Mullershausen *et al.*, 2004; Bawankule *et al.*, 2005; Teixeira *et al.*, 2006a,b).

The purpose of the present study was to clarify the mechanisms of action of BAY 41-2272 in inhibiting the aggregation of washed rat platelets and to further assess the interactions of this compound with NO donor drugs and with a stable prostacyclin analogue, beraprost.

Methods

This study complied with the National Research Council *Guide for the Care and Use of Laboratory Animals,* as well as the guidelines established by the ethical committee of the Institut de Recherches Servier.

Washed platelet preparation

Male Wistar Kyoto (WKY) rats (12-week-old; Charles River, l'Arbresle, France) were anesthetized with pentobarbital sodium (50 $mg \cdot kg^{-1}$ intraperitoneally). One carotid artery was catheterized and the blood was drawn with a syringe and collected in sodium citrate (0.109 M, one volume of citrate for nine volumes of blood). The whole blood was centrifuged at room temperature (2300× *g*, for 2 min at 20°C) and the supernatant, the platelet-rich plasma, was collected and again centrifuged (530× *g*, for 15 min at 20°C). The platelet-poor plasma was removed and the platelet pellet was suspended in 10 mL Tyrode solution of the following composition (mM) NaCl 137; KCl 2.7; $NaHCO_3$ 11.9; $MgCl_2$1.0; NaH_2PO_4 0.34; HEPES 5.0; glucose 5.6) in the absence of calcium but in the presence of prostaglandin E_1 (0.1 μM), apyrase (0.5 $U \cdot mL^{-1}$) and albumin (0.35%). This platelet suspension was re-centrifuged (530× *g*, for 15 min at 20°C) and the platelet pellet was again suspended in Tyrode solution, now containing calcium (2 mM), in the presence of apyrase and albumin. Platelet count was performed (Beckman Coulter) and the washed platelet count was adjusted to 300 000 platelets μL^{-1} by adding appropriate volumes of calcium containing Tyrode solution.

Platelet aggregation

Platelet aggregation was performed with an optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, the Netherlands) at 37°C with 250 μL of washed platelets placed in glass cuvettes containing a disposable stir bar for constant stirring (1000 r.p.m.). Before stirring, drugs [ODQ, L-nitro-arginine (L-NA), hydroxocobalamin] or solvents were incubated for 10 min prior to the addition of BAY 41-2272, sodium nitroprusside, nitroglycerin, DEA-NONOate or/and beraprost (10 min incubation) and then aggregation was elicited by adenosine 5′-diphosphate (ADP), collagen or thrombin and followed for 8 min. Stirring was started 2 min before the addition of the aggregating agent. The maximal aggregation (%) and the area-under-the-curve parameter were calculated using the Aggrolink software (Chrono-log). Tyrode solution with or without drug solvents, depending on the experimental protocols, provided a signal representing 0% aggregation. In order to allow for reversible aggregation, the parameter selected to analyse the data obtained in the various protocols was the area under the curve.

In preliminary experiments, it was established that ADP (0.1–30 μM) produced a dose-dependent aggregation of rat platelets (data not shown). The lowest dose producing a maximal aggregation was identified for subsequent experiments, i.e. 10 μM ADP. Also, BAY 41-2272 (0.1–10 μM) produced a concentration-dependent and virtually complete inhibition of the aggregation elicited by ADP (10 μM), collagen (5 $\mu g \cdot mL^{-1}$) or thrombin (5 $U \cdot mL^{-1}$) (IC_{50}: 1.9, 1.7 and 5.9 μM respectively; n = 5–6), while the three NO donors, DEA-NONOate, sodium nitroprusside and nitroglycerin, produced only a partial inhibition of ADP- and collagen-induced platelet aggregation and virtually no effect of those elicited by thrombin. Therefore, the subsequent experiments presented in this study were performed using ADP (10 μM) as an aggregating agent.

cGMP and cAMP measurements

cGMP and cAMP formation was determined in washed platelets (300 000 platelets per μL in Tyrode buffer, at 37°C) simultaneously with aggregation. The platelet preparation was incubated in the glass cuvette for 10 min with or without the various inhibitors studied and without stirring. Then NO donors, BAY 41-2272 and/or beraprost were added, and the incubation was continued further for 8 min. ADP (10 μM) was then added, stirring having been started 2 min before the addition of ADP. The reactions were stopped at the end of the aggregation process (10 min after the addition of ADP) by adding to the cuvettes an equal volume of ice-cold solution containing ethanol 90% and HCl (0,1N) 10%. The samples were vortexed for 30 sec and kept in ice before centrifugation (1500× g for 30 min at 4°C). The supernatants (400 μL) were collected in glass tubes and evaporated in a Speed Vac for 45 min before being re-suspended in assay buffer. cGMP and cAMP levels were measured using commercially available cGMP and cAMP enzyme immunoassay kits according to the instructions of the manufacturer (Cayman Chemical, SPI-Bio, Ann Arbor, MI, USA).

Data analysis

Data are shown as mean ± SEM; n indicates the number of rats from which platelets were collected. Comparisons versus control were performed statistically using a one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test for paired or unpaired experiments or by use of Student's t-test for paired or unpaired observations, as appropriate. In most experimental protocols, experiments were performed in parallel and are considered as paired experiments; that is, the washed platelets from the same animal are subjected to different treatments. Differences were considered to be statistically significant when the P-value was less than 0.05.

Materials

Adenosine 5′-diphosphate, ODQ, isobutyl-methyl-xanthine (IBMX), L-NA, hydroxocobalamin, sodium nitroprusside, thrombin (Sigma, La Verpillère, France); nitroglycerin (Merck, Darmstadt, Germany); DEA-NONOate (Alexis Chemical, Coger, Paris France); beraprost (Cayman Chemical, Ann Arbor, MI, USA); collagen (Kordia, Leiden, the Netherlands). The compound (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine) (BAY 41-2272) was synthesized at the Institut de Recherches Servier (Suresnes, France). The compounds were dissolved in distilled water at the exception of IBMX, ODQ and BAY 41-2272, which were daily prepared as stock solution in dimethyl sulphoxide (DMSO) and further diluted in water. For each protocol, water and/or DMSO were added in each cuvette (volumes of 2.5 μL), so that the concentrations of solvents were the same in any given experimental condition.

Results

BAY 41-2272 and cGMP production in washed platelets of Wistar Kyoto rats

In unstimulated washed platelets of WKY rats, BAY 41-2272 (10 μM) and the non-specific phosphodiesterase inhibitor, IBMX (0.5 mM), produced a similar time-dependent increase in cGMP that levelled off 10 min after the administration of either compound. The presence of BAY 41-2272 produced a marked potentiation of the IBMX-induced increase in cGMP (Figure 1). The increase in cGMP production elicited by BAY 41-2272 was inhibited by the presence of ODQ (5 μM) over the whole duration of the experiment. Thus, the evaluation of cGMP generation at the end of the aggregation process (18 min) could be considered as a reasonable estimate of the exposure to cGMP when the aggregation process is elicited with ADP (at 10 min). Although the presence of the non-specific inhibitor of phosphodiesterases IBMX enhanced the levels of cGMP, increased levels of this cyclic nucleotide can reasonably be achieved in the absence of phosphodiesterase inhibition, allowing simultaneous measurements of cyclic nucleotide levels and aggregation. Therefore, in the subsequent experiments presented in this study, the measurement of cGMP was made, in the absence of IBMX, at the end of the aggregation process elicited by ADP (10 μM), that is, 8 min after the addition of ADP.

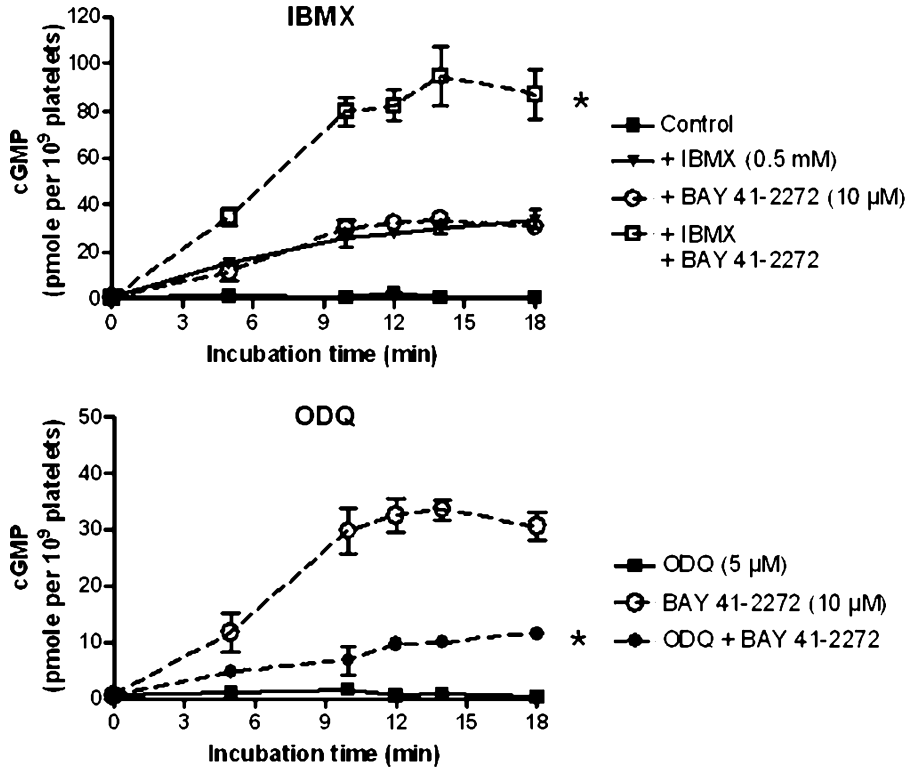

Figure 1

BAY 41-2272 (10 μM) and time-dependent increases in cGMP levels in unchallenged WKY washed platelets. Upper panel: with or without IBMX (0.5 mM). Lower panel: with or without ODQ (5 μM). Data are shown as mean ± SEM. $N = 4$, number of rats from which the platelets were collected. *$P < 0.05$; significant effect of either IBMX or ODQ (for paired experiments). BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; IBMX, isobutyl methyl xanthine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; WKY, Wistar Kyoto.

Interactions between BAY 41-2272 and nitric oxide

BAY 41-2272 (0.1–10 μM) produced a concentration-dependent inhibition of the aggregation elicited by ADP, which was associated with a concentration-dependent increase in cGMP production (Figure 2).

The NO synthase inhibitor, L-NA (500 μM), did not affect ADP-induced platelet aggregation or the basal level of cGMP. The guanylyl cyclase inhibitor, ODQ (5 μM), did not affect ADP-induced aggregation but significantly reduced basal cGMP levels, while the NO scavenging agent, hydroxocobalamin (100 μM), significantly enhanced platelet aggregation and decreased basal cGMP levels (Table 1). In the presence of either ODQ or hydroxocobalamin, the inhibition of the aggregation and the increase in cGMP produced by BAY 41-2272 were significantly inhibited. In contrast, in the presence of L-NA, the responses elicited by the soluble guanylyl cyclase stimulator were unaffected (Figures 2 and 3).

Sodium nitroprusside and nitroglycerin (1–100 μM) partially inhibited platelet aggregation, a phenomenon associated with an increase in cGMP. Both were abolished by the presence of ODQ. However, the NO donor DEA-NONOate (1–100 μM) produced a concentration-dependent and partial inhibition of the ADP-induced aggregation that was not significantly affected by the presence of ODQ, although the guanylyl cyclase inhibitor significantly reduced the associated increase in cGMP (Figure 4). In contrast, both effects of the three NO donors (10 μM) were inhibited by the presence of hydroxocobalamin (Figure 5).

The presence of a sub-maximal concentration of each of the three NO donors (3 μM) potentiated a minimally effective concentration of BAY 41-2272 (0.3 μM)-induced cGMP generation and inhibition of platelet aggregation (Figure 6).

Interactions between BAY 41-2272 and the prostacyclin pathway

Beraprost (10 nM–1 μM), the stable analogue of prostacyclin, produced a concentration-dependent inhibition of ADP-induced platelet aggregation associated with a marked increase in cAMP generation

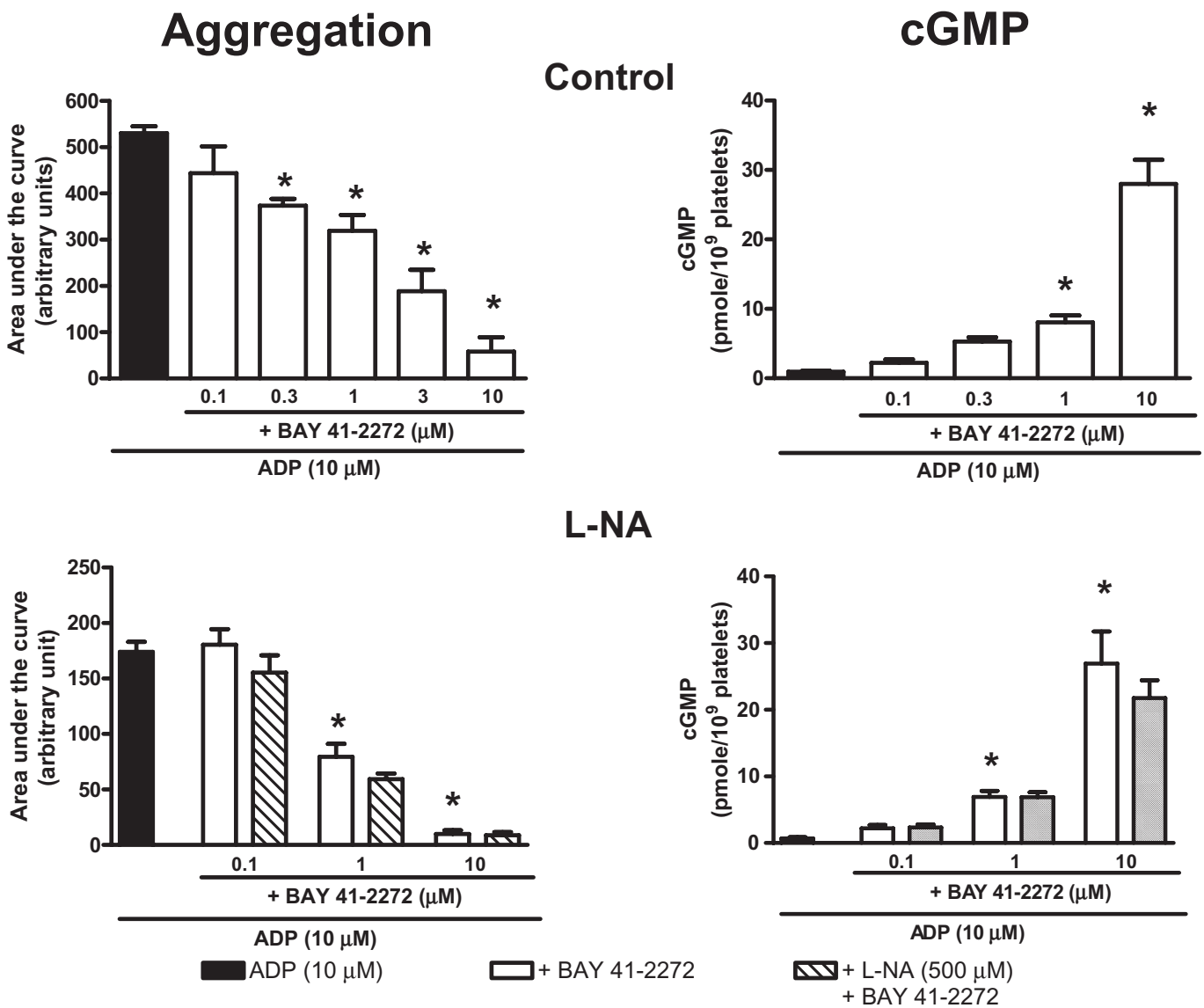

Figure 2

Effects of BAY 41-2272 in WKY washed platelets. Upper panels: BAY 41-2272 induces concentration-dependent inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panel, $n = 3–6$). The associated changes in cGMP levels are shown on the right panel ($n = 4–20$). Lower panels: Effect of L-NA (500 μM) on BAY 41-2272 (0.1, 1 and 10 μM) producing inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panel, $n = 10–14$). The associated changes in cGMP levels are shown on the right panel ($n = 10–14$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. *$P < 0.05$; significant effect of BAY 41-2272 (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; WKY, Wistar Kyoto.

but with no significant changes in the levels of cGMP (Figure 7).

BAY 41-2272 (0.3 and 1 μM) produced a significant increase in basal cAMP levels (Table 2). The presence of this compound influenced the changes in cAMP levels evoked by beraprost in a biphasic manner. Thus, the increase in cAMP levels generated by 10 nM beraprost was enhanced in the presence of BAY 41-2272 at the low concentration of 0.3 μM. At a higher concentration of beraprost (30 nM), the levels of cAMP were not affected by the presence of BAY 41-2272, but at the higher concentration of 1 μM, it significantly reduced the cAMP production generated by 100 nM beraprost (Table 3). In contrast, beraprost (10, 30 or 100 nM) did not affect the changes in cGMP induced by BAY 41-2272 (Figure 8 and Table 3).

When compared with beraprost alone, the combination of beraprost (30 nM) plus BAY 41-2272 (0.3 μM), a treatment that did not enhance the

Table 1

Intrinsic effects of L-nitroarginine (L-NA: 500 μM), ODQ (5 μM) and hydroxocobalamin (100 μM) on ADP (10 μM)-induced aggregation and cGMP generation in rat washed platelets

	Aggregation amplitude (%)	Aggregation AUC (arbitrary unit)	cGMP (pmole per 10^9 platelets)
Control	63 ± 2, *n* = 13	201 ± 11, *n* = 13	1.03 ± 0.21, *n* = 13
+L-NA	60 ± 1, *n* = 15	174 ± 10, *n* = 15	0.77 ± 0.15, *n* = 13
Control	76 ± 4, *n* = 16	493 ± 37, *n* = 16	1.19 ± 0.35, *n* = 8
+ODQ	77 ± 3, *n* = 16	492 ± 37, *n* = 16	0.25 ± 0.08*, *n* = 8
Control	63 ± 1, *n* = 20	197 ± 11, *n* = 20	1.23 ± 0.16, *n* = 20
+Hydroxocobalamin	67 ± 1*, *n* = 20	274 ± 14*, *n* = 20	0.43 ± 0.07*, *n* = 20

*$P < 0.05$; significant effect of treatments (for paired or unpaired experiments). Data are shown as mean ± SEM. *N* indicates the number of rats from which platelets were harvested.
ADP, adenosine 5′-diphosphate; L-NA, L-nitro-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

cAMP production induced by beraprost, potentiated the anti-aggregating effects (Figure 8).

Hydroxocobalamin (100 μM) prevented the increase in cAMP and cGMP produced by BAY 41-2272 (0.3 and 1 μM) but did not affect the increase in cAMP elicited by beraprost (30 and 100 nM) (Table 2 and Figure 8). This NO scavenger prevented the potentiation produced by BAY 41-2272 of the anti-aggregating effect of beraprost (Figure 8).

Discussion

The present study shows that, in washed rat platelets, BAY 41-2272, a stimulator of soluble guanylyl cyclase is a potent inhibitor of platelet aggregation, but only in the presence of NO.

The anti-aggregating effects of DEA-NONOate, sodium nitroprusside and nitroglycerin, three NO donors, were associated with cGMP production. These compounds produce only a partial inhibition of the aggregation induced by ADP and are much less potent in inhibiting platelet aggregation than in producing vascular relaxation in isolated arterial rings of the same species, confirming previous observations in rat platelets (Homer and Wanstall, 2002, and data not shown). The cGMP production in response to DEA-NONOate was consistently higher than that observed in response to the two other NO donors, most likely because cGMP generation is related to the rate of spontaneous release of NO from the donor compound (Gordge *et al.*, 1998). Nevertheless, ODQ inhibited the cGMP generated by the three NO donors, but abolished the anti-aggregating effects of only two of them, sodium nitroprusside and nitroglycerin. The anti-aggregator effect of DEA-NONOate was not affected by ODQ, although cGMP generation was virtually abolished. This observation confirms previously published data with NO donors that generate NO spontaneously and it has been suggested that this type of NO donor would inhibit platelet aggregation in a cGMP-independent manner (Sogo *et al.*, 2000; Wanstall *et al.*, 2005). However, in genetically modified mice with a deletion of the β_1-subunit of the soluble guanylyl cyclase, which leads to the complete loss of soluble guanylyl cyclase activity, there is no evidence for such a cGMP-independent effects of NO donors (Dangel *et al.*, 2010). One possible explanation is that in rodent platelets, NO donors produced a rapid rise in cGMP, within the first few seconds after their administration, and this early rise in cGMP is likely to play an important role in inhibiting platelet aggregation (Salvemini *et al.*, 1990; Mo *et al.*, 2004). As NO and ODQ compete at the same site on soluble guanylyl cyclase, the haem iron, differences in the rate of NO release may differently affect the inhibitory property of ODQ. The rapid NO release, characteristic of DEA-NONOate, may not allow a proper inhibition of soluble guanylyl cyclase by ODQ in the first seconds following its administration while a complete inhibition can be achieved a few minutes later; that is, in the present study, when the cGMP levels were measured. Hydroxocobalamin, the NO scavenger (Rochelle *et al.*, 1995), abolished both the cGMP generation and the anti-aggregating effects of sodium nitroprusside and nitroglycerin and only partially inhibited those to DEA-NONOate, again most likely because the rapid NO release, characteristic of the latter compound, exceeded the scavenging capacity of hydroxocobalamin. Taken together, these data indicate that the effects of the three compounds can indeed be attributed to NO production.

The anti-aggregating effects of BAY 41-2272 were also associated with cGMP production. The marked

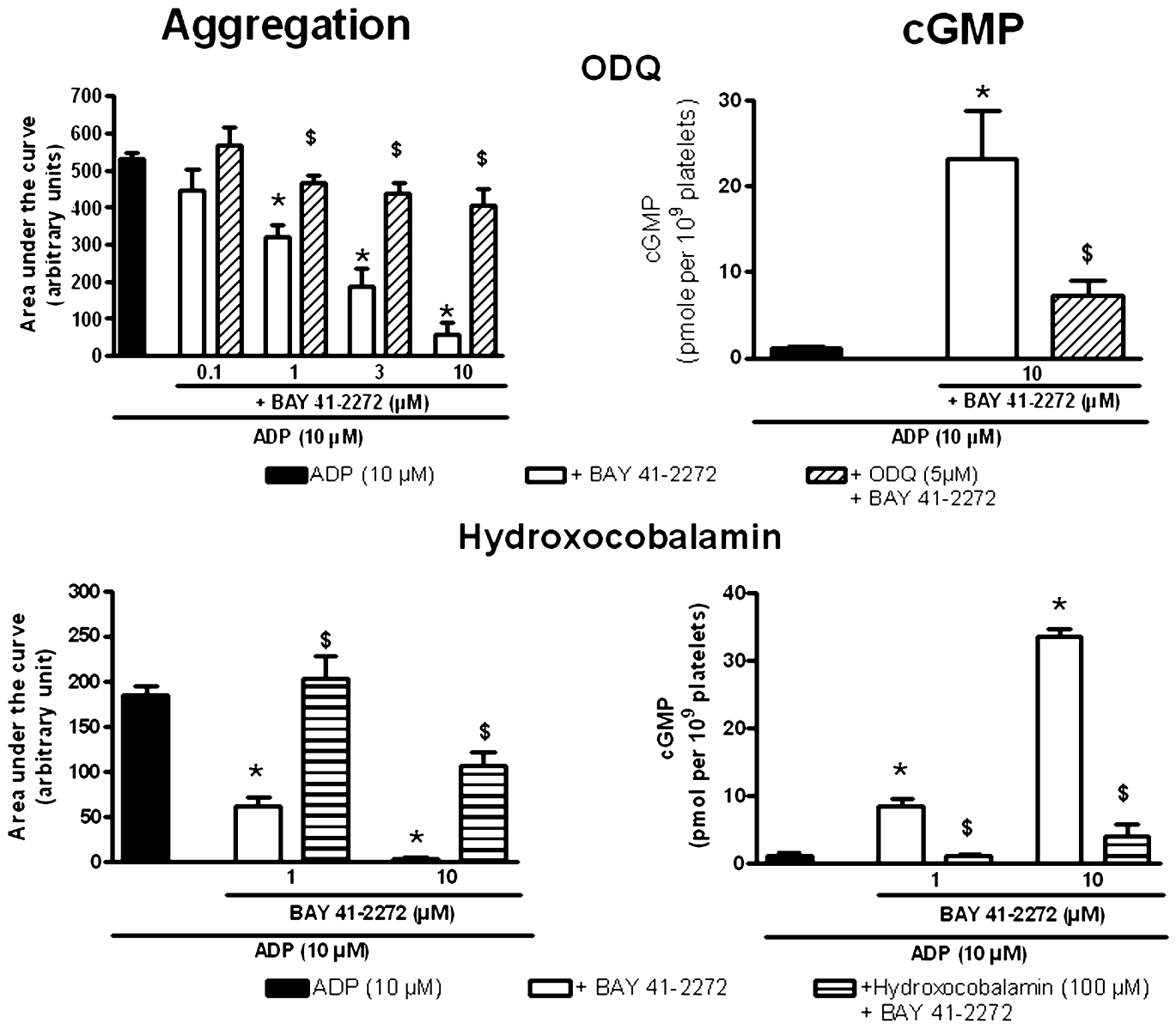

Figure 3

Effects of BAY 41-2272 in WKY washed platelets. Upper panels: Effect of an inhibitor of soluble guanylyl cyclase, ODQ (5 μM), on BAY 41-2272 producing concentration-dependent inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panel, $n = 3–6$). The associated changes in cGMP levels produced by 10 μM of BAY 41-2272 are shown on the right panel ($n = 4$). Lower panels: Effect of a NO scavenger, hydroxocobalamin (100 μM), on BAY 41-2272 (1 and 10 μM) producing inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panel, $n = 3–6$). The associated changes in cGMP levels are shown on the right panel ($n = 3–6$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. $^{*}P < 0.05$; significant effect of BAY 41-2272. $^{\$}P < 0.05$; significant effect of ODQ or hydroxocobalamin (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NO, nitric oxide; WKY, Wistar Kyoto.

potentiation by BAY 41-2272 of IBMX-induced increase in cGMP levels suggests that, under our experimental conditions, phosphodiesterase inhibition, a putative ancillary property of BAY 41-2272 (Mullershausen *et al.*, 2004), is not its preponderant mechanism of action.

As expected, the exposure of platelets to ODQ, which induces haem oxidation and/or increases the rate of haem dissociation from the soluble guanylyl cyclase, prevented BAY 41-2272-induced inhibition of platelet aggregation and the associated increase in cGMP production (Garthwaite *et al.*, 1995; Roy *et al.*, 2008a,b; Stasch *et al.*, 2006). Hydroxocobalamin prevented the generation of cGMP elicited by BAY 41-2272 and the associated inhibition of platelet aggregation, indicating that, in rat platelets, BAY

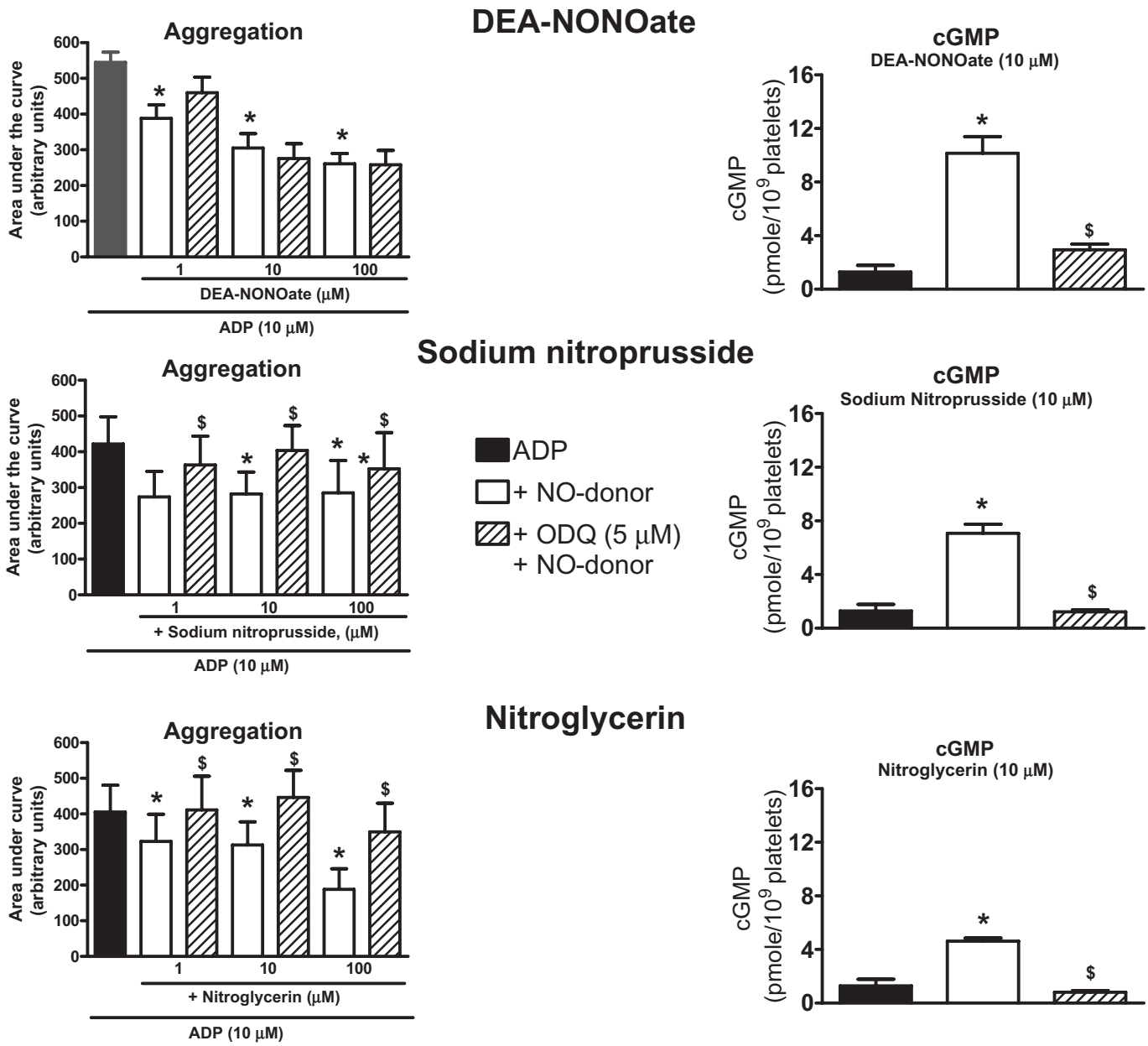

Figure 4

Effects of NO donors in WKY washed platelets. Effect of ODQ (5 μM) on the three NO donors, DEA-NONOate, sodium nitroprusside and nitroglycerin, producing concentration-dependent inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panels, $n = 5–7$). The associated changes in cGMP levels produced by 10 μM of DEA-NONOate, sodium nitroprusside or nitroglycerin are shown on the right panels ($n = 5$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. $^{*}P < 0.05$; significant effect of DEA-NONOate, sodium nitroprusside or nitroglycerin. $^{\$}P < 0.05$; significant effect of of ODQ (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; DEA-NONOate, 2-(N,N-diethylamino)-diazenolate 2-oxide sodium salt hydrate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NO, nitric oxide; WKY, Wistar Kyoto.

41-2272 required the presence of NO to produce its effect. Both ODQ and hydroxocobalamin decreased basal cGMP levels indicating that, under our experimental conditions, NO was present in the preparation of washed platelets and that it stimulated the soluble guanylyl cyclase. However, the NO synthase inhibitor L-NA, even at the elevated concentration of 500 μM, did not affect either basal levels of cGMP or those generated by BAY 41-2272, indicating that, in the preparation of WKY washed platelets, NO synthases are not the source of NO. Whether or not functional NO synthase(s) are present in platelets has been a matter of controversy over the years. Recent evidence obtained, either *in vitro* or *in vivo*, in

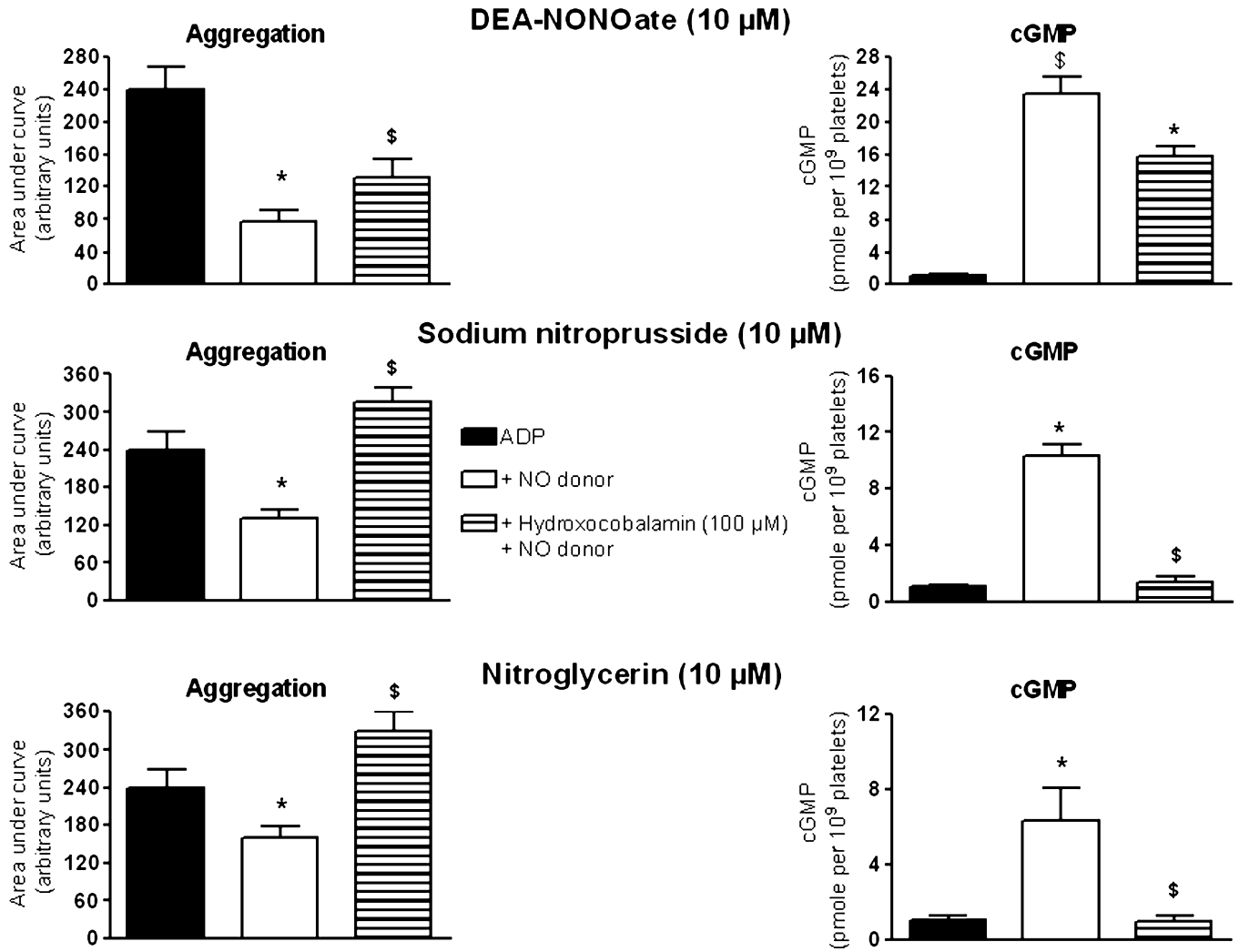

Figure 5

Effects of NO donors in WKY washed platelets. Effect of hydroxocobalamin (100 μM) on the three NO donors, DEA-NONOate, sodium nitroprusside and nitroglycerin (10 μM), producing inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panels, $n = 6$). The associated changes in cGMP levels are shown on the right panels ($n = 6$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. *$P < 0.05$; significant effect of DEA-NONOate, sodium nitroprusside or nitroglycerin. $^{\$}P < 0.05$; significant effect of hydroxocobalamin (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; DEA-NONOate, 2-(N,N-diethylamino)-diazenolate 2-oxide sodium salt hydrate; NO, nitric oxide; WKY, Wistar Kyoto.

rodent and human platelets, along with the data from the present study, are not in favour of a major role of platelet NO synthase(s) in regulating their aggregation (Gambaryan *et al.*, 2008; Naseem and Riba, 2008; Tymvios *et al.*, 2009). Nevertheless, our observations are consistent with the model developed by Roy *et al.* (2008b) indicating that BAY 41-2272 is a pure allosteric enhancer of soluble guanylyl cyclase activity, which has no or very little activity when steps are taken to exclude NO. In the presence of BAY 41-2272, the potency of NO becomes extremely high, in the low picomolar range, a concentration which is well within the range expected to exist in solutions exposed to environmental air (Roy *et al.*, 2008b; Garthwaite, 2010). Therefore, under the present experimental conditions, the origin of NO could be the 'contamination' produced by the traces of the gas contained in the atmosphere. Whether BAY 41-2272 is truly an NO-independent soluble guanylyl cyclase stimulator (Stasch and Hobbs, 2009), which, in the absence of NO, is not potent enough to produce a measurable increase in cGMP and the inhibition of platelet aggregation, or whether the presence of traces of NO are absolutely required but have been overlooked in earlier studies, remains to be determined. Nevertheless, the result of this work shows that at least traces of NO have to be present in order to

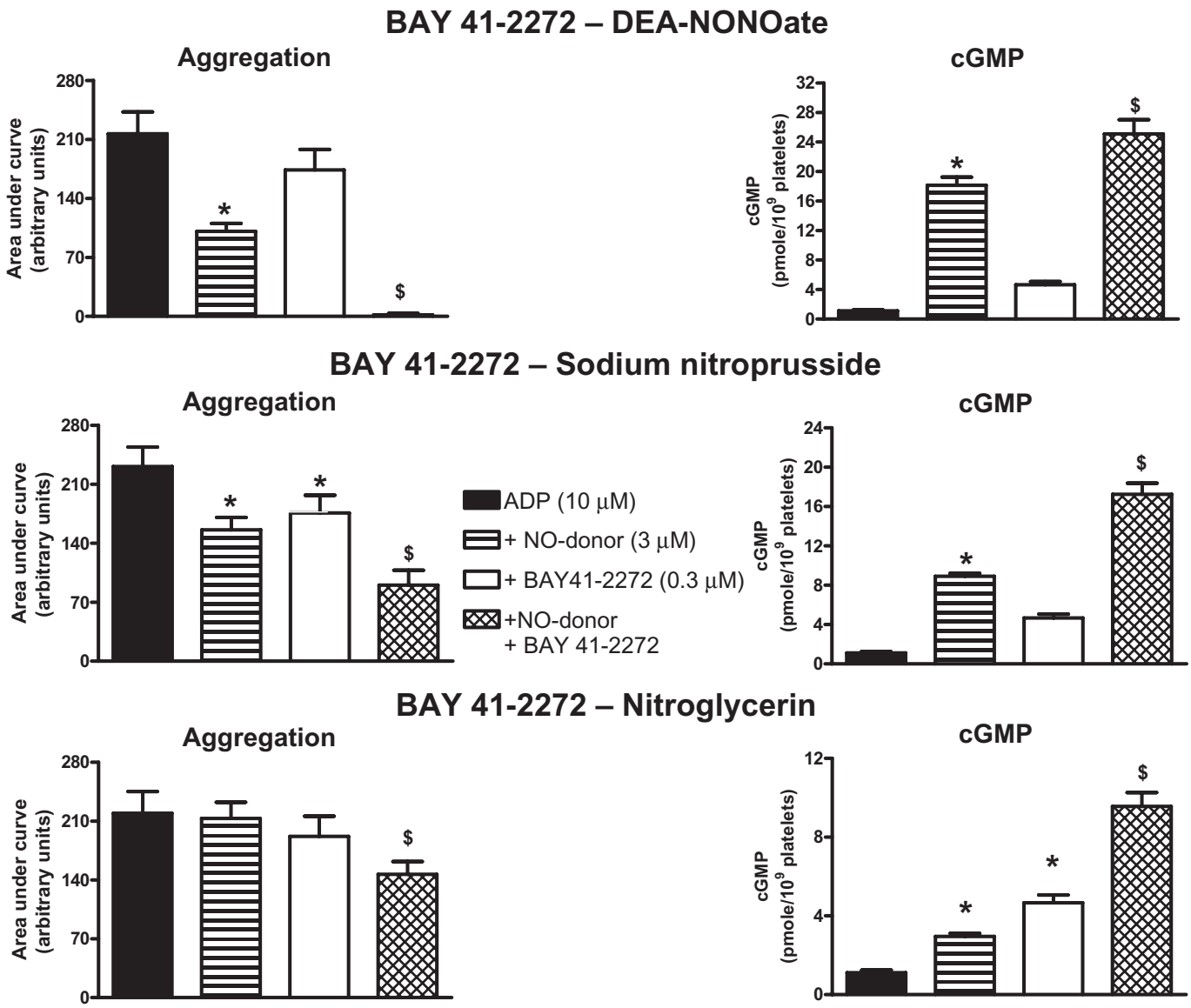

Figure 6

Effects of BAY 41-2272 and NO donors in WKY washed platelets. The effects of the interactions between BAY 41-2272 (0.3 μM) and the three NO donors DEA-NONOate, sodium nitroprusside and nitroglycerin (3 μM) on ADP (10 μM)-induced aggregation are shown on the left panels (n = 12). The associated changes in cGMP levels are shown on the right panels (n = 6). Data are shown as mean ± SEM. N indicates the number of rats from which the platelets were collected. *P < 0.05; significant effect of either BAY 41-2272 or a given NO donor (DEA-NONOate, sodium nitroprusside or nitroglycerin). $^{\$}P$ < 0.05; significantly different from the effect of the most potent of either of BAY 41-2272 or a given NO donor, taken individually (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; DEA-NONOate, 2-(N,N-diethylamino)-diazenolate 2-oxide sodium salt hydrate; NO, nitric oxide; WKY, Wistar Kyoto.

observe a measurable anti-aggregating effect of BAY 41-2272.

In the present study, the specificity of hydroxocobalamin could be assumed as it inhibited both the increase in cGMP production and the inhibitory effects produced by the three different NO donors. Furthermore, this NO scavenger did not influence the increase in cAMP production or the anti-aggregating effects elicited by beraprost. Finally and more importantly, hydroxocobalamin did not affect the increase in cGMP production and the anti-aggregating effects elicited by BAY 58-2667, a truly NO-independent activator of soluble guanylyl cyclase (see Figure S1). In the present study, the observed inhibitory effect of hydoxocobalamin on BAY 41-2272 is in contrast to the reported effect of another NO scavenger, haemoglobin, which did not influence the cGMP production and the inhibition

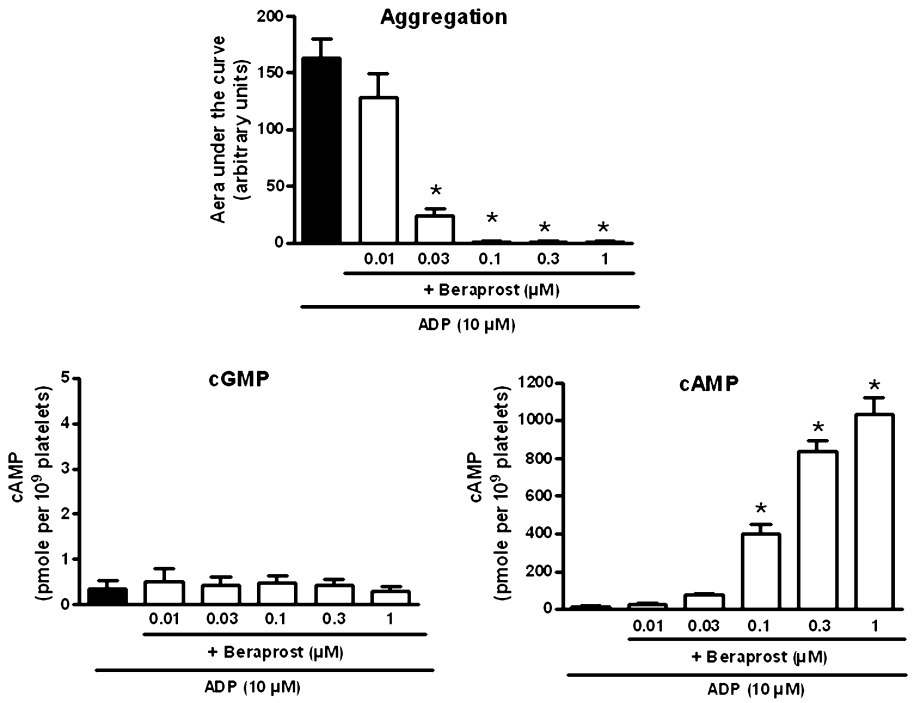

Figure 7

Effects of beraprost in WKY washed platelets. Beraprost (0.01 to 1 µM), a synthetic agonist of the IP receptor, induces inhibition of ADP (10 µM)-induced aggregation of WKY washed platelets (upper panel, $n = 6$). The associated changes in cGMP and cAMP levels are shown on the lower panels ($n = 5$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. *$P < 0.05$; significant effect of beraprost ($P < 0.05$, for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; WKY, Wistar Kyoto.

Table 2

Effects of hydroxocobalamin (100 µM) on the changes in cAMP (pmole per 10^9 platelets) induced by BAY 41-2272 or beraprost in rat washed platelets (in the presence of ADP: 10 µM)

	Control	+ Hydroxocobalamin
ADP	30 ± 2	29 ± 1
BAY 41-2272 (0.3 µM)	39 ± 2*	28 ± 1$^{\$}$
+BAY 41-2272 (1 µM)	42 ± 3*	30 ± 1$^{\$}$
+Beraprost (30 nM)	89 ± 15*	99 ± 12*
+Beraprost (100 nM)	359 ± 38*	396 ± 33*

*$P < 0.05$; significant effect of BAY 41-2272 or beraprost; $^{\$}P < 0.05$; significant effect of hydroxocobalamin (for paired experiments). Data are shown as mean ± SEM. $N = 6$, number of rats from which platelets were collected.
ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)].

of aggregation elicited by another soluble guanylyl cyclase stimulator, YC-1 (Ko *et al.*, 1994). Therefore, in platelets, YC-1 appears to be a NO-independent stimulator of soluble guanylyl cyclase, its mechanism of action being somehow different from that of BAY 41-2272. As these two compounds are structurally related, this interpretation appears unlikely (Stasch *et al.*, 2001). However, YC-1 is much less specific than BAY 41-2272 and among its major side effects is inhibition of phosphodiesterases (Galle *et al.*, 1999; Stasch and Hobbs, 2009; Garthwaite, 2010). As the inhibition of platelet aggregation by phosphodiesterase inhibitors is not affected by the presence of NO scavengers (Salvemini *et al.*, 1990), this ancillary property of YC-1 may explain the discrepancies mentioned above.

A positive interaction was observed between the three NO donors and the stimulator of soluble guanylyl cyclase BAY 41-2272 and the changes in cGMP were correlated to the enhanced inhibition of platelet aggregation. However, the combination of BAY 41-2272 plus DEA-NONOate was more efficient than the combination of BAY 41-2272 with the two other NO donors. Even when the concentration of sodium nitroprusside and nitroglycerin was raised up to 30 µM, the addition of BAY 41-2272 did not produce a complete inhibition of the aggregation (data not shown). Again, this increased potency is more likely to be attributed to the fact that DEA-NONOate is a rapid NO releasing compound (being a more potent agent in raising

Table 3

Effects of BAY 41-2272 on the changes in cGMP and cAMP induced by beraprost, in rat washed platelets (in the presence of ADP: 10 μM)

	n	cGMP (pmole per 10^9 platelets)	cAMP (pmole per 10^9 platelets)
Control	11	0.8 ± 0.3	23 ± 3
+BAY 41-2272 (0.3 μM)	11	4.7 ± 0.8*	33 ± 3*
+BAY 41-2272 (1 μM)	6	10.6 ± 2.5*	42 ± 3*
Beraprost (10 nM)	5	0.5 ± 0.3	25 ± 6
Beraprost + BAY 41-2272 (0.3 μM)	5	3.3 ± 0.9*	35 ± 6*
Beraprost (30 nM)	11	1.4 ± 0.4	83 ± 9
Beraprost + BAY 41-2272 (0.3 μM)	11	4.8 ± 1.1*	94 ± 8
Beraprost (100 nM)	11	1.2 ± 0.3	378 ± 30
Beraprost + BAY 41-2272 (1 μM)	6	9.2 ± 3.2*	257 ± 26*

*$P < 0.05$; significant effect of BAY 41-2272 (for paired or unpaired experiments). Data are shown as mean ± SEM. *N* indicates the number of rats from which platelets were collected.
ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)].

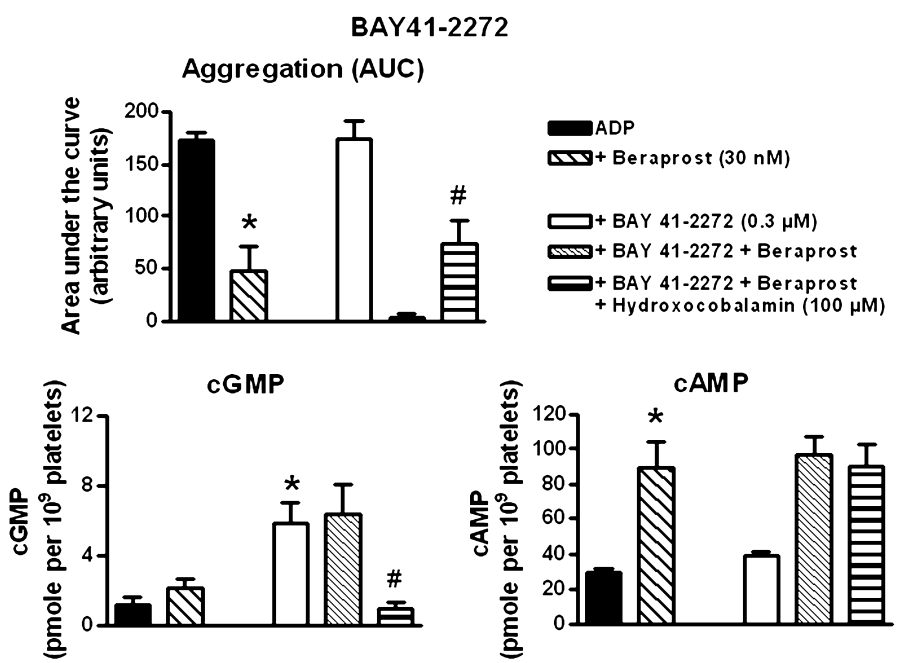

Figure 8

Effects of BAY 41-2272 and beraprost in WKY washed platelets. Effects of hydroxocobalamin (100 μM) on the interactions between BAY 41-2272 (0.3 μM) and beraprost (30 nM) in WKY washed platelets. The changes in ADP (10 μM)-induced aggregation are shown on the top panel and the associated changes in cGMP and cAMP levels are shown on the bottom panels ($n = 6$). Data are shown as mean ± SEM. *N* indicates the number of rats from which the platelets were collected. *$P < 0.05$; significant effect of either BAY 41-2272 or beraprost. $^{\#}P < 0.05$, significant effect of hydroxocobalamin on the combination of BAY 41-2272 plus beraprost (for paired or unpaired experiments). ADP, adenosine 5′-diphosphate; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine)]; WKY, Wistar Kyoto.

cGMP levels than either sodium nitroprusside or nitroglycerin) than to the contribution of undetermined cGMP-independent effects.

Beraprost is a synthetic prostacyclin analogue, which in rat platelets inhibits aggregation by activating the IP prostaglandin receptor (Sim *et al.*, 1985; Gomez *et al.*, 2008; nomenclature follows Alexander *et al.*, 2009). In platelets, the IP receptor is predominantly coupled to the adenylyl cyclase pathway (Smyth and FitzGerald, 2002) and indeed the potent anti-aggregating effect of beraprost was associated with concentration-dependent increases

in cAMP (Walter *et al.*, 1993). BAY 41-2272 did also increase, although to a much lesser extent than beraprost, cAMP levels. Hydroxocobalamin inhibited both the increase in cGMP and cAMP produced by BAY 41-2272. Therefore, BAY 41-2272 is likely to produce a secondary increase in cAMP following the cGMP-dependent inhibition of phosphodiesterase-3 and the resultant decrease in cAMP breakdown, as it has been shown for various nitrovasodilators and another stimulator of soluble guanylyl cyclase (Maurice and Haslam, 1990; Wu *et al.*, 1995; Anfossi *et al.*, 2001; Feijge *et al.*, 2004). In the combined presence of a low concentration of beraprost with BAY 41-2272, the observed increases in cAMP are consistent with a purely additive effect of these two compounds. In the presence of higher concentrations of beraprost, this additive effect disappears and paradoxically in the presence of a combination of an elevated concentration of beraprost plus an elevated concentration of BAY 41-2272, the cAMP levels were significantly lower than in the presence of beraprost alone. The inhibitory effect of BAY 41-2272 on cAMP levels could be attributed to the cGMP-dependent activation of phosphodiesterase-2, which, in platelets and for high cAMP concentrations, plays a major role in the metabolism of cAMP (Dickinson *et al.*, 1997).

The present study confirms that BAY 41-2272 synergizes with an IP receptor agonist (Hobbs and Moncada, 2003). These observations are in agreement with the synergy reported in platelet preparations between NO donors and prostacyclin or its analogues (Lidbury *et al.*, 1989; Negrescu *et al.*, 1995; Dickinson *et al.*, 1997). However, the present study shows that this synergy can occur without reciprocal changes in cyclic nucleotides; that is, the increase in cAMP produced by beraprost was not affected by the presence of BAY 41-2272 and similarly the increase in cGMP produced by the presence of BAY 41-2272 was not influenced by beraprost. Hydroxocobolamin did not influence the effects of beraprost confirming that the effects of beraprost are NO-independent. However, hydroxocobolamin inhibited the increase in cGMP and the potentiating effect of BAY 41-2272 on beraprost-induced inhibition of platelet aggregation. These data are in agreement with previous observations showing that activated protein kinase A and protein kinase G can phosphorylate the same targets, for instance VASP or rap1b, and increase synergistically their activities to a level much higher than the sum of their individual actions (Walter *et al.*, 1993; Reep and Lapetina, 1996).

In conclusion, the present study confirms that, in washed rat platelets, the stimulator of soluble guanylyl cyclase, BAY 41-2272, inhibits platelet aggregation and that this effect is associated with the generation of both cGMP and cAMP. BAY 41-2272 requires the reduced form of soluble guanylyl cyclase and the obligatory presence of NO. The positive interaction observed between BAY 41-2272 and various NO donors is qualitatively similar whatever the mechanism involved in NO release. Furthermore, a potent synergism is observed between BAY 41-2272 and prostacyclin analogues, but again only if traces of NO are present.

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Conflict of interests

None.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Anfossi G, Russo I, Massucco P, Mattiello L, Balbo A, Cavalot F *et al.* (2001). Studies on inhibition of human platelet function by sodium nitroprusside. Kinetic evaluation of the effect on aggregation and cyclic nucleotide content. Thromb Res 102: 319–330.

Bawankule DU, Sathishkumar K, Sardar KK, Chanda D, Krishna AV, Prakash VR *et al.* (2005). BAY 41-2272 [5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine]-induced dilation in ovine pulmonary artery: role of sodium pump. J Pharmacol Exp Ther 314: 207–213.

Bhardwaj R, Page CP, May GR, Moore PK (1988). Endothelium-derived relaxing factor inhibits platelet aggregation in human whole blood in vitro and in the rat in vivo. Eur J Pharmacol 157: 83–91.

Dangel O, Mergia E, Karlisch K, Groneberg D, Koesling D, Friebe A (2010). NO-sensitive guanylyl cyclase is the only NO receptor mediating platelet inhibition. J Thromb Haemost 8: 1343–1352.

Dickinson NT, Jang EK, Haslam RJ (1997). Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation. Biochem J 323: 371–377.

Evgenov OV, Pacher P, Schmidt PM, Haskó G, Schmidt HH, Stasch J-P (2006). NO-independent stimulators and activators of soluble guanylyl cyclase: discovery and therapeutic potential. Nat Rev Drug Discov 5: 755–768.

Feijge MA, Ansink K, Vanschoonbeek K, Heemskerk JW (2004). Control of platelet activation by cyclic AMP turnover and cyclic nucleotide phosphodiesterase type-3. Biochem Pharmacol 67: 1559–1567.

Furchgott RF (1988). Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte PM (ed.). Mechanism of Vasodilatation. Raven Press: New York, pp. 401–414.

Furchgott RF, Zawadzki JV (1980). The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373–376.

Galle J, Zabel U, Hübner U, Hatzelmann A, Wagner B, Wanner C *et al*. (1999). Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. Br J Pharmacol 127: 195–203.

Gambaryan S, Kobsar A, Hartmann S, Birschmann I, Kuhlencordt PJ, Müller-Esterl W *et al*. (2008). NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity. J Thromb Haemost 6: 1376–1384.

Garthwaite J (2010). New insight into the functioning of nitric oxide-receptive guanylyl cyclase: physiological and pharmacological implications. Mol Cell Biol 334: 221–232.

Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. Mol Pharmacol 48: 184–188.

Gomez E, Schwendemann C, Roger S, Simonet S, Paysant J, Courchay C *et al*. (2008). Aging and prostacyclin responses in aorta and platelets from WKY and SHR rats. Am J Physiol Heart Circ Physiol 295: H2198–H2211.

Gordge MP, Hothersall JS, Noronha-Dutra AA (1998). Evidence for a cyclic GMP-independent mechanism in the anti-platelet action of S-nitrosoglutathione. Br J Pharmacol 124: 141–148.

Hobbs AJ, Moncada S (2003). Antiplatelet properties of a novel, non-NO-based soluble guanylate cyclase activator, BAY 41-2272. Vascul Pharmacol 40: 149–154.

Homer KL, Wanstall JC (2002). Inhibition of rat platelet aggregation by the diazeniumdiolate nitric oxide donor MAHMA NONOate. Br J Pharmacol 137: 1071–1081.

Ignarro LJ (1991). Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. Blood Vessels 28: 67–73.

Ignarro LJ, Byrns RE, Wood KS (1988). Biochemical and pharmacological properties of EDRF and its similarity to nitric oxide radical. In: Vanhoutte PM (ed.). Mechanism of Vasodilatation. Raven Press: New York, pp. 427–435.

Ko FN, Wu CC, Kuo SC, Lee FY, Teng CM (1994). YC-1, a novel activator of platelet guanylate cyclase. Blood 84: 4226–4233.

Lidbury PS, Antunes E, de Nucci G, Vane JR (1989). Interactions of iloprost and sodium nitroprusside on vascular smooth muscle and platelet aggregation. Br J Pharmacol 98: 1275–1280.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S *et al*. (2000). Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev 52: 375–414.

Maurice DH, Haslam RJ (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. Mol Pharmacol 37: 671–681.

Mo E, Amin H, Bianco IH, Garthwaite J (2004). Kinetics of a cellular nitric oxide/cGMP/phosphodiesterase-5 pathway. J Biol Chem 18: 26149–26158.

Moncada S, Palmer RJM, Higgs EA (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43: 109–142.

Mullershausen F, Russwurm M, Friebe A, Koesling D (2004). Inhibition of phosphodiesterase type 5 by the activator of nitric oxide-sensitive guanylyl cyclase BAY 41-2272. Circulation 109: 1711–1713.

Naseem KM, Riba R (2008). Unresolved roles of platelet nitric oxide synthase. J Thromb Haemost 6: 10–19.

Negrescu EV, Grünberg B, Kratzer MA, Lorenz R, Siess W (1995). Interaction of antiplatelet drugs in vitro: aspirin, iloprost, and the nitric oxide donors SIN-1 and sodium nitroprusside. Cardiovasc Drugs Ther 9: 619–629.

Radomski MW, Palmer RMJ, Moncada S (1987). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol 92: 639–646.

Rapoport RM, Murad F (1983). Agonist induced endothelium-dependent relaxation in rat thoracic aorta may be mediated though cyclic GMP. Circ Res 52: 352–357.

Reep BR, Lapetina EG (1996). Nitric oxide stimulates the phosphorylation of rap1b in human platelets and acts synergistically with iloprost. Biochem Biophys Res Commun 219: 1–5.

Rochelle LG, Morana SJ, Kruszyna H, Russell MA, Wilcox DE, Smith RP (1995). Interactions between hydroxocobalamin and nitric oxide (NO): evidence for a redox reaction between NO and reduced cobalamin and reversible NO binding to oxidized cobalamin. J Pharmacol Exp Ther 275: 48–52.

Roy B, Mo E, Vernon J, Garthwaite J (2008a). Probing the presence of the ligand-binding haem in cellular nitric oxide receptors. Br J Pharmacol 153: 1495–1504.

Roy B, Halvey EJ, Garthwaite J (2008b). An enzyme-linked receptor mechanism for nitric oxide-activated guanylyl cyclase. J Biol Chem 283: 18841–18851.

Salvemini D, Radziszewski W, Korbut R, Vane J (1990). The use of oxyhaemoglobin to explore the events underlying inhibition of platelet aggregation induced by NO or NO-donors. Br J Pharmacol 101: 991–995.

Schmidt HH, Schmidt PM, Stasch J-P (2009). NO- and haem-independent soluble guanylate cyclase activators. Handb Exp Pharmacol 191: 309–339.

Sim AK, McCraw AP, Cleland ME, Nishio S, Umetsu T (1985). Effect of a stable prostacyclin analogue on platelet function and experimentally-induced thrombosis in the microcirculation. Arzneimittelforschung 35: 1816–1818.

Smyth EM, FitzGerald GA (2002). Human prostacyclin receptor. Vitam Horm 65: 149–165.

Sogo N, Magid KS, Shaw CA, Webb DJ, Megson IL (2000). Inhibition of human platelet aggregation by nitric oxide donor drugs: relative contribution of cGMP-independent mechanisms. Biochem Biophys Res Commun 279: 412–419.

Stasch J-P, Hobbs AJ (2009). NO-independent, haem-dependent soluble guanylate cyclase stimulators. Handb Exp Pharmacol 191: 277–308.

Stasch J-P, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A *et al.* (2001). NO-independent regulatory site on soluble guanylate cyclase. Nature 410: 212–215.

Stasch J-P, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, Arun Kumar HS, Meurer S *et al.* (2006). Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. J Clin Invest 116: 2552–2261.

Teixeira CE, Priviero FB, Todd J Jr, Webb RC (2006a). Vasorelaxing effect of BAY 41-2272 in rat basilar artery: involvement of cGMP-dependent and independent mechanisms. Hypertension 47: 596–602.

Teixeira CE, Priviero FB, Webb RC (2006b). Molecular mechanisms underlying rat mesenteric artery vasorelaxation induced by the nitric oxide-independent soluble guanylyl cyclase stimulators BAY 41-2272 [5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b] pyridin-3-yl]pyrimidin-4-ylamine] and YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl Indazole. J Pharmacol Exp Ther 317: 258–266.

Tymvios C, Moore C, Jones S, Solomon A, Sanz-Rosa D, Emerson M (2009). Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. Br J Pharmacol 158: 1735–1742.

Walter U, Eigenthaler M, Geiger J, Reinhard M (1993). Role of cyclic nucleotide-dependent protein kinases and their common substrate VASP in the regulation of human platelets. Adv Exp Med Biol 344: 237–249.

Wanstall JC, Homer KL, Doggrell SA (2005). Evidence for, and importance of, cGMP-independent mechanisms with NO and NO donors on blood vessels and platelets. Curr Vasc Pharmacol 3: 41–53.

Wu CC, Ko FN, Kuo SC, Lee FY, Teng CM (1995). YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. Br J Pharmacol 116: 1973–1978.

Supporting information

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

Figure S1

Beraprost and BAY 58-2667 in WKY washed platelets: Influence of the presence of hydroxocobalamin (100 μM). Top panels: Beraprost (0.03 to 0.1 μM), a synthetic agonist of the IP receptor, induces inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left). The associated changes in cGMP and cAMP levels are shown on the centre and right panels respectively. Bottom panels: BAY 58-2667 or Cinaciguat (0.01 to 1 μM), a truly NO-independent activator of soluble guanylyl cyclase, induces inhibition of ADP (10 μM)-induced aggregation of WKY washed platelets (left panel). The associated changes in cGMP and cAMP levels are shown on the centre and right panels respectively. Data are shown as mean ± SEM. n = 6 and indicates the number of rats from which the platelets were harvested.

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