The synergism of hydrogen peroxide with plasma S-nitrosothiols in the inhibition of platelet activation

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Earlier studies have shown that inhibition of aggregation of washed platelets (WP) by NO was enhanced almost 100-fold by \( \text{H}_2\text{O}_2 \). In the present study, the interactions of \( \text{H}_2\text{O}_2 \) with nitrosothiols, the influence of the presence of plasma and the mechanism of the synergism were investigated. \( \text{H}_2\text{O}_2 \) strongly enhanced the inhibitory effects of S-nitrosoglutathione (GSNO) on thrombin-induced aggregation of WP. S-Nitrosoalbumin also inhibited platelets, and this was similarly enhanced by \( \text{H}_2\text{O}_2 \). The synergism with \( \text{H}_2\text{O}_2 \) was demonstrable for both exogenous GSNO and NO in the presence of plasma when platelets were stimulated with collagen. The inhibition of platelets by GSNO and \( \text{H}_2\text{O}_2 \) was completely inhibited by guanylly cyclase inhibitors. Synergism was also observed whether the \( \text{H}_2\text{O}_2 \) was added simultaneously or 1 min before or after the GSNO (or NO). This suggests that the action of \( \text{H}_2\text{O}_2 \) follows the occupation by NO of haem sites in guanylate cyclase and that a prior reaction between NO and \( \text{H}_2\text{O}_2 \) was not required. In the absence of exogenous GSNO or NO, \( \text{H}_2\text{O}_2 \) inhibited activation of platelets in plasma, an effect abolished by guanylly cyclase inhibitors. This suggested that endogenous NO donors in plasma or NO synthesized in platelets may interact with \( \text{H}_2\text{O}_2 \). Addition of \( \text{N}^\omega\)-nitro-\( \text{L}\)-arginine methyl ester (hydrochloride) (\( \text{L}\)-NAME) decreased the effects of the \( \text{H}_2\text{O}_2 \) by 25\%, indicating that the major endogenous source of NO in platelet-rich plasma was not derived from platelet synthesis of NO but from NO donors in plasma, such as nitrosothiols. Inhibition by \( \text{H}_2\text{O}_2 \) was also enhanced by \( \beta\)-mercaptosuccinate, a glutathione peroxidase inhibitor that protects the \( \text{H}_2\text{O}_2 \). These results suggest a potent synergism of \( \text{H}_2\text{O}_2 \) with endogenous plasma nitrosothiols that inhibit platelet function through an intracellular mechanism involving guanylate cyclase.

INTRODUCTION
NO is synthesized from \( \text{L}\)-arginine by the enzymic action of NO synthases [1]. NO enters adjacent cells, where it binds to the haem moiety of soluble guanylate cyclase, leading to the formation of cGMP, which induces smooth muscle cell relaxation and platelet inhibition [2]. S-Nitrosothiols, such as S-nitrosocysteine and S-nitrosoglutathione (GSNO), may be formed by the S-nitrosation of free thiol groups in the presence of NO [3] by oxygen-dependent processes [4,5], but this is still a matter of debate. Alternatively, nitrosothiols may be formed by reaction of thiols with peroxynitrite, which is derived from the reaction of NO with superoxide anion [6]. More recently, it was suggested that GSNO could be formed intracellularly from glutathione and organic nitrates by the action of microsomal glutathione S-transferase [7,8]. The nitrosothiols have differing degrees of stability, but have been shown to have biological properties similar to those of NO, which may be released from them. S-Nitrosothiols induce arterial relaxation [9] and inhibit platelet aggregation \( \textit{in vitro} \) [10,11] and \( \textit{in vivo} \) [12], with associated increases in cGMP. Thiol groups on proteins are also nitrosated and are relatively stable. The full biological significance of S-nitrosothiols remains unclear, but may represent a mechanism by which NO can exert a more systemic effect or act as a sink to prolong the action of NO intracellularly. 

\( \text{H}_2\text{O}_2 \) is produced by dismutation of superoxide anion (\( \text{O}_2^- \)) [13]. It has been shown to induce endothelial-dependent relaxations of bovine pulmonary arteries [14] and enhanced NO synthase activity in rabbit aortic rings [15]. \( \text{H}_2\text{O}_2 \) in the absence of other agonists, induces primary aggregation of washed platelets (WP), and acts synergistically with other platelet agonists [16], but paradoxically has also been shown to inhibit platelet function when added before the agonists [17]. Both NO and \( \text{H}_2\text{O}_2 \) are synthesized during platelet activation [18,19]. However, in a recent study it was shown that, when added simultaneously to stirred WP, there was a profound synergy between NO and \( \text{H}_2\text{O}_2 \) so that the potency of NO as an inhibitor of platelet aggregation increased almost 100-fold [20]. These inhibitory actions of mixtures of NO and \( \text{H}_2\text{O}_2 \) were associated, at least in part, with increases in platelet cGMP. However, the site of this interaction was unclear. In the present study, the inhibitions with authentic NO solutions were compared with those of a simple nitrosothiol, GSNO, and S-nitrosoalbumin as a representative nitrosated protein. The aim was to determine whether the interactions with \( \text{H}_2\text{O}_2 \) could occur in plasma with nitrosothiols, increasing the likely biological relevance of the earlier findings. It was important to determine whether these interactions could occur within the platelets, since NO derived from nitrosothiols is likely to be released intracellularly [12]. Furthermore, it is important to determine whether these interactions occur only when the NO (or NO donor) and \( \text{H}_2\text{O}_2 \) are added simultaneously to the platelets. Further information is required on the mechanism of this synergism.

MATERIALS AND METHODS
\( \text{H}_2\text{O}_2 \), sodium urate, prostacyclin (synthetic sodium salt), thrombin (human), glutathione, \( \text{N}^\omega\)-nitro-\( \text{L}\)-arginine methyl ester (hydrochloride) (\( \text{L}\)-NAME), dibutyryl cGMP (dBCGMP), isobutyl-

Abbreviations used: GSNO, S-nitrosoglutathione; DTPA, diethylenetriaminepenta-acetic acid; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide; dBCGMP, dibutyryl cGMP; IC\(_{50}\), concentration required to produce inhibition of 50% ; LY88585, 6-amino-5,8-
quinolinoquinone; ODQ, oxadiazoloquinoxaline-1-one; \( \text{L}\)-NAME, \( \text{N}^\omega\)-nitro-\( \text{L}\)-arginine methyl ester (hydrochloride); PRP, platelet-rich plasma; WP, washed platelets.

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methylxanthine, N-ethylmaleimide and β-mercaptosuccinate were all purchased from Sigma Chemicals Co. (Poole, Dorset, U.K.). Collagen type I (equine) was from Hormonchemie (Munich, Germany). NO gas was from Lynde Gas (Stoke-on-Trent, U.K.). GSNO and oxadiazoloquinoxalin-1-one (ODQ), guanylate cyclase inhibitors, were from Tocris-Cookson Chemicals (Southampton, U.K.). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide (carboxy-PTIO) and 6-aminino-5,8-quinolinequinone (LY85835) were from Calbiochem-Novabiochem (Nottingham, U.K.).

Platelet preparations

Venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days. Blood was taken into acid/citrate/dextrose anticoagulant and centrifuged for 20 min at 150 g to yield platelet-rich plasma (PRP), which was used within 3 h. For the preparation of WP, the PRP was recentrifuged in the presence of 50 nM prostacyclin at 800 g for 10 min to obtain a platelet pellet [21]. This was suspended in physiological buffer (150 mM NaCl, 5 mM Hepes, 0.55 mM NaHPO₄, 7 mM NaHCO₃, 2.7 mM KCl, 0.5 mM MgCl₂, 5.6 mM glucose, pH 7.4) and diluted to a count of 3 × 10⁶ platelets/ml. The platelets were left for 1 h at room temperature to recover their sensitivity to aggregating agents. In addition, platelets were prepared from PRP by lowering the pH to 6.5, centrifuging and restoring the pH to 7.4, as previously described [20], to study these interactions on very fresh platelets.

NO, GSNO, S-nitrosoalubmin and H₂O₂ solutions

NO was prepared as solutions based on an established method [22] and as previously described [20]. The concentrations of stock solutions of NO were checked with the Griess reaction and with an NO electrode (World Precision Instruments, Stevenage, U.K.). Fresh solutions of GSNO were prepared in deoxygenated water for each experiment. S-Nitrosoalubmin was prepared by incubation of human serum albumin with acidified sodium nitrite, and the degree of nitrosation was determined using the Griess reaction [23]. H₂O₂ was diluted from a stock solution (8.82 M) into deoxygenated water and kept sealed in air-tight vials throughout the experiments. Controls for the peroxide were prepared by exposing the H₂O₂ to air overnight or boiled for 60 min before dilution.

Platelet aggregation tests

Platelet aggregation tests were performed using nephelometry [24]. WP or PRP were incubated for 1 min at 37 °C with continuous stirring at 1000 rev./min in a Payton Dual-channel aggregometer (Centronic Sales Ltd., Croydon, U.K.) before addition of agonists or antagonists. Platelet aggregation was induced by addition of either collagen (0.5 µg/ml final concentration) or thrombin (0.02 unit/ml) and measured as the increase in light transmittance after 3 min. NO, GSNO or S-nitrosoalubmin, either in combination with H₂O₂ (simultaneously unless otherwise stated) or with buffer, were added to platelets 1 min before the addition of the agonist. Each compound was delivered via a separate syringe.

Measurement of NO and GSNO concentrations in the presence of H₂O₂

Stock solutions of NO were prepared as described above. The NO electrode was kept at equilibrium with air in a vessel containing continually stirred Tyrode’s Heps buffer, pH 7.4 (20 ml). Aliquots of NO or GSNO solutions were added to the buffer to give final concentrations of 20 nM NO or GSNO, and the concentrations were determined against a series of NO standards generated from acidified sodium nitrite. The changes in concentration over several minutes were measured in the presence and absence of 20 µM H₂O₂.

Radioimmunoassay for cGMP

The platelets were treated with agonists and antagonists at the appropriate interval of time, in the presence of isobutyl-methylxanthine (200 µM). The incubation was stopped by the addition of 1 M HCl, and the radioimmunoassay was performed with kits purchased from Amersham International (Amersham, Bucks., U.K.). All data are expressed as the means ± S.E.M. of three to five independent experiments unless stated otherwise. Statistical evaluation was by unpaired Student’s t test. The IC₅₀ values of individual experiments were calculated from concentration-response curves created using the curve-fitting option of the scientific graph software Fig. P. (Biosoft, Ferguson, U.S.A.).

RESULTS

Inhibition of platelet aggregation by NO and GSNO

Authentic NO solutions added as a bolus induced a concentration-dependent inhibition of thrombin-induced aggregation (IC₅₀ 95 ± 40 nM) of WP with complete inhibition achieved at 3.3 ± 1.8 µM (Figure 1). GSNO (0.01–10 µM) also inhibited thrombin-induced aggregation of WP in a concentration-dependent manner and was more potent than NO: the IC₅₀ was 19.3 ± 2.4 nM (P < 0.05) and complete inhibition was attained at 133 ± 47 nM (P < 0.01) (Figure 1).

Carboxy-PTIO reacts with NO stoichiometrically (carboxy-PTIO/NO = 1.0) in neutral solution, antagonizing the biological effects of NO [25]. Pre-incubation of carboxy-PTIO (1 µM) with platelets, 1 min before the addition of NO, significantly decreased the inhibitory actions of NO (Figure 1). For example, NO

![Figure 1](image-url)
(100 nM) inhibited aggregation by 52.5±2.4%, but in the presence of carboxy-PTIO (1 µM) this was reduced to 2.5±2.2%. In contrast, carboxy-PTIO at this concentration did not affect platelet inhibition induced by GSNO. The IC₅₀ values for GSNO in the presence and absence of carboxy-PTIO were 24 and 26 nM respectively (Figure 1), indicating that GSNO releases NO intracellularly or at a site not available to carboxy-PTIO.

Simultaneous addition of GSNO and H₂O₂ to WP

H₂O₂ strongly enhanced the inhibitory actions of GSNO in WP, as was previously shown with NO (Figure 2, top). Simultaneous addition of GSNO (0.0001–1 µM) and H₂O₂ (20 µM) resulted in a concentration-dependent inhibition of thrombin-induced aggregation: the IC₅₀ for GSNO in the presence of H₂O₂ was 0.71±0.21 nM, over 27-fold lower than for GSNO alone (P<0.01) (Figure 2, top). The concentration of GSNO at which complete inhibition was achieved was also lower (16.7±2.1 nM) than in the absence of H₂O₂ (P<0.05) (Figure 2, top). Reduced glutathione (100 nM) used as a control for GSNO did not affect platelet aggregation (Figure 2, top). When carboxy-PTIO was added to mixtures of GSNO and H₂O₂, there was no diminution of the synergy between the two reagents (Figure 2, top), suggesting an intracellular interaction.

In order to explore the concentration at which H₂O₂ was effective, the experiments were repeated with a fixed concentration of GSNO (10 nM) and H₂O₂ from 0.01–20 µM (Figure 2, middle). The EC₅₀ for H₂O₂ was 2.7±0.3 µM, and inhibition was maximal at 20 µM (96.2±2.1%). The term EC₅₀ (effective concentration at 50%) is used to describe the actions of H₂O₂, since it increased the action of GSNO. Enhancement of GSNO-mediated inhibition was not observed with H₂O₂ solutions that had been either left exposed overnight or heated at 100 °C for 30 min (Figure 2, middle).

Pre-mixing GSNO and H₂O₂ for up to 1 min before addition to platelets showed little effect on the synergism with respect to inhibition of platelets (results not shown). GSNO (10 nM) alone induced 24.8±6.2% inhibition when added directly to WP; this remained unchanged if the GSNO was pre-mixed with buffer for 1 min, stirred at 37 °C, GSNO (10 nM) and H₂O₂ (20 µM) added simultaneously and directly to platelets induced 94.2±4.3% inhibition, and again this was unchanged if the reagent were premixed for 45 s before addition to the platelets. Premixing for 1 min led to a small reduction in the level of inhibition (86.8±3.6%, n=3), but this was not statistically significant (results not shown).

A significant proportion of the plasma nitrosothiols reside in the proteins [3]. The effects of S-nitrosoalbumin (0.4 mol of NO/mol of human serum albumin) were therefore investigated; this had a similar inhibitory effect to GSNO and NO on the activation of WP (IC₅₀ 70±18 nM) (Figure 2, bottom). Furthermore, the S-nitrosoalbumin also acted synergistically with 20 µM H₂O₂, which decreased significantly the IC₅₀ to 15±7.5 nM (P<0.05, n=4).

It has been reported that H₂O₂ enhances the activity of cyclooxygenase in a number of cell types; therefore the synergism of GSNO and H₂O₂ was also investigated in platelets that had been pretreated with 100 µM aspirin for 30 min. This had no effect on inhibition by GSNO and H₂O₂ of thrombin-stimulated platelets (results not shown), and the efficacy of the aspirin was demonstrated by their failure to respond to collagen (1 g/l). Also, platelets that had been isolated by lowering the pH, rather than by adding prostacyclin, gave identical results with GSNO or S-nitrosoalbumin and H₂O₂, as was observed previously with authentic NO and H₂O₂ [20].
The influence of H$_2$O$_2$ on inhibition by NO and GSNO in PRP

Collagen-induced aggregation of PRP was inhibited by pre-incubation for 1 min with either NO (0.01–10 µM) or GSNO (0.01–10 µM), although greater concentrations of these inhibitors were required than those for WP. The IC$_{50}$ values were 826 ± 168 nM and 477 ± 162 nM for NO and GSNO respectively (Figure 3): these inhibitors were approx. 20-fold less effective in PRP than in WP. Mixtures of NO (0.01–10 µM) and H$_2$O$_2$ (1–500 µM) inhibited aggregation induced by collagen to a greater extent than NO alone, the IC$_{50}$ falling to 149 ± 66 nM (Figure 3, left panel). The synergism between GSNO and H$_2$O$_2$ was also observed in PRP. GSNO (0.01–10 µM) and H$_2$O$_2$ added simultaneously to PRP inhibited aggregation induced by collagen to a greater extent than GSNO alone (Figure 3, right panel). The IC$_{50}$ for GSNO in the presence of H$_2$O$_2$ (20 µM) was reduced 15-fold from 477 ± 162 nM to 32 ± 9 nM ($P < 0.05$). The addition of S-nitrososalbumin (molar ratio 0.4 mol of NO/mol of albumin) also inhibited platelet aggregation in PRP, and the effects were enhanced by the addition of H$_2$O$_2$ (results not shown).

If H$_2$O$_2$ was added to PRP 1 min before the addition of collagen, but in the absence of exogenous NO or GSNO, it had an anti-aggregatory effect of its own at concentrations of 10 µM or above (Tables 1 and 2). This did not occur in WP, suggesting that the H$_2$O$_2$ was acting synergistically with endogenous NO donors. The inhibitory action of H$_2$O$_2$ was enhanced by addition of β-mercaptosuccinate (10 µM), an inhibitor of plasma glutathione peroxidase, which reduces the peroxide, and also to a slightly lesser extent by N-ethylmaleimide, another inhibitor of the peroxidase (Table 1). If the PRP was pre-incubated with L-NAME (100 µM) for 5 min to inhibit platelet NO synthesis, then the effects of H$_2$O$_2$ were slightly reduced, suggesting that the principal NO source was from an NO donor in the plasma (Table 2). The concentrations of L-NAME used followed those employed in platelet cytosol in the presence of 300 µM l-arginine to cause near-maximal inhibition of NO synthase after 3 min [26]. At higher concentrations of L-NAME (800 µM) and with longer pre-incubation times with this inhibitor (15 min), no greater reduction in the H$_2$O$_2$-induced inhibition of platelet aggregation was achieved (results not shown). Incubation of WP with β-mercaptosuccinate enhanced the inhibition of aggregation by GSNO alone, suggesting that accumulation of endogenous H$_2$O$_2$ can also play a role in the synergism (results not shown) and indicating a role for the platelet glutathione peroxidase in regulating platelet H$_2$O$_2$ release.

### Table 1

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<th>Addition</th>
<th>H$_2$O$_2$ (µM)</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
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<td>21 ± 4.5</td>
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<tr>
<td>β-Mercaptosuccinate</td>
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<td>41 ± 7.5*</td>
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<tr>
<td>N-Ethylmaleimide</td>
<td>20</td>
<td>43 ± 9*</td>
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### Table 2

<table>
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<th>Inhibition (%)</th>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4 ± 2.1</td>
</tr>
<tr>
<td>10</td>
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<td>20</td>
<td>38 ± 3.7</td>
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<tr>
<td>L-NAME (100 µM)</td>
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</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2.5 ± 1.7</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>12 ± 2.1*</td>
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<tr>
<td>20</td>
<td>20</td>
<td>24 ± 4*</td>
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Figure 3 Influence of NO or GSNO and H$_2$O$_2$ on platelet aggregation in the presence of plasma

Left: PRP was incubated with NO (0.001–10 µM) in the presence (●) or absence (○) of H$_2$O$_2$ for 1 min before the addition of collagen (0.5 µg/ml), and aggregation was measured 3 min later. In all cases the H$_2$O$_2$ was added to the platelets simultaneously with NO. The results are expressed as the percentages of inhibition of platelet aggregation and represent the means ± S.E.M. of four independent experiments. Right: as for the left panel except that GSNO was used instead of NO, in the presence (●) and absence of H$_2$O$_2$ (○).
Effects of radical scavengers on GSNO and GSNO/H2O2-induced inhibition

In order to investigate further the mechanism of the interaction of GSNO and H2O2, the platelets were pre-incubated with the radical scavengers sodium urate (0.5 mM) and mannitol (50 mM) for 1 min with the WP. These scavengers had no effect on the inhibition of platelet activation by GSNO or GSNO/H2O2. However, if urate or mannitol was incubated with the isolated platelets for 30 min before addition of GSNO/H2O2, the subsequent inhibition of thrombin-induced aggregation was attenuated. The level of inhibition was reduced modestly from 92.5 ± 1.1 % (buffer control) to 75.9 ± 4 % for urate (P < 0.05, n = 3) and to 80 ± 3.3 % (P < 0.05, n = 3) in the case of mannitol. The scavengers had no influence on inhibition by GSNO alone and had no effect on platelet activation at the concentrations used. The effects of these scavengers on authentic NO and H2O2 were greater and occurred within 1 min [20].

Influence of H2O2 on the stability of NO and GSNO in solution

The stability of dilute solutions of NO or GSNO may be followed continuously using an NO electrode, and it was possible to ascertain whether H2O2 influences the rate of these reagents, at least exogenously, to the platelet. The addition of H2O2 had only a slight effect on the decay of the NO (less than 10 %; results not shown). GSNO slowly released NO in solution, which was also measured with the electrode. The rate of appearance of NO was not changed by addition of H2O2 (results not shown), indicating that neither the release of NO from GSNO nor the decomposition of NO was influenced significantly by H2O2.

Importance of the time intervals separating the addition of GSNO or NO and H2O2 on the inhibition of platelet aggregation

The necessity for simultaneous addition of the NO source and H2O2 for the synergy to occur was investigated. The effects of NO and GSNO were compared in this respect. The addition of NO or GSNO was kept constant at 1 min before addition of thrombin (0.02 unit/ml) to WP, but H2O2 (20 μM) was added up to 1 min before or after addition of GSNO or NO (both 10 mM). Simultaneous addition of GSNO/H2O2 induced 92.2 ± 2.1 % inhibition of aggregation. However, if H2O2 was added before GSNO addition, a reduction in the level of inhibition was observed (Figure 4, left panel). Incubation of H2O2 with platelets for 1 min before GSNO addition induced an inhibition of 61.1 ± 6 % (P < 0.01 compared with the simultaneous addition of the reagents), whereas incubation for 30 s beforehand induced an inhibition of 72.2 ± 1.1 % (P < 0.01). Addition of H2O2 15 s before GSNO addition also slightly reduced the inhibition, but this was not significant compared with simultaneous additions (Figure 4, left panel). This indicated that the effects of H2O2 were attenuated, probably due to the reduction in the peroxide concentrations because of the activity of platelet glutathione peroxidase before addition of GSNO. If H2O2 was added after GSNO for up to 1 min, no significant change in the level of inhibition was observed (Figure 4, left panel).

When these experiments were repeated with NO instead of GSNO, the effect of separation of addition of NO and H2O2 was greater, with a clear optimal synergism when the two were added simultaneously. However, the synergistic interactions were still observable at all the time intervals studied (Figure 4, left panel).

Role of cGMP on the interaction of GSNO with H2O2

When guanylate cyclase activity in platelets was inhibited by the addition of 10 μM LY85835, the inhibition of platelet activity by GSNO over a range of concentrations was almost completely blocked (results not shown). The inhibitor had no effect on platelet activation in the absence of GSNO. When GSNO (0–100 nM) was added simultaneously with H2O2, the inhibition was attenuated by 90 ± 3 % (results not shown), suggesting that the mechanism of GSNO/NO interaction with H2O2 is directly associated with or dependent on guanylate cyclase. Incubation of WP with another guanylate cyclase inhibitor, ODQ, also led to complete abolition of the inhibition of platelet activity by both GSNO and GSNO added with H2O2 (results not shown). In
addition, the inhibitory actions of H$_2$O$_2$ alone on collagen-induced aggregation in PRP were completely abolished by pre-incubation of platelets with the soluble guanylate cyclase inhibitors LY83835 or ODQ (results not shown).

The effect of H$_2$O$_2$ on platelet inhibition downstream of guanylate cyclase was also investigated. Incubation of WP with dbcGMP for 1 min before the addition of thrombin led to a concentration-dependent inhibition of aggregation (Figure 5). The IC$_{50}$ for dbcGMP was 220 ± 30 µM, and complete inhibition was achieved at 2 mM. Addition of H$_2$O$_2$ (25 µM) in combination with dbcGMP (0–5 mM) led to an enhancement of the inhibitory actions of dbcGMP. The IC$_{50}$ for dbcGMP in the presence of H$_2$O$_2$ was reduced to 100 ± 10 µM (P < 0.05), whereas complete inhibition was unchanged at 2 mM. The magnitude of the change was relatively modest in comparison with the effects of H$_2$O$_2$ on the action of NO and GSNO.

As indicated above, the interaction of NO and H$_2$O$_2$ is partly dependent on the time when the two mediators are added to the platelets, with optimal inhibitory effects obtained with simultaneous addition (Figure 4). In this experiment, cGMP concentrations were measured in parallel incubations in the presence of a phosphodiesterase inhibitor. The extent of the inhibition of aggregation, at these time points of separation of NO and H$_2$O$_2$, was paralleled to a significant degree by variations in the aggregations were measured in parallel incubations in the presence of platelets, with optimal inhibitory effects obtained with sim-

DISCUSSION

GSNO has a biological half-life on platelets several times longer than authentic NO [22]. The synthesis of both NO and H$_2$O$_2$ is reported to be greater in platelets activated by collagen compared with thrombin [26,27]. The inhibition of activation of WP by GSNO was enhanced considerably by H$_2$O$_2$, but to a slightly lesser extent than reported earlier for authentic NO and this peroxyde [20]. This synergism occurred in WP at very low concentrations of GSNO (less than 1 nM) and at physiologically relevant concentrations of H$_2$O$_2$. The interaction of GSNO and H$_2$O$_2$ was not inhibited by an excess of carboxy-PTIO (unlike the interactions of NO and H$_2$O$_2$), indicating that these interactions occur intracellularly. Similar interactions on the inhibition of platelet aggregation were also observed between H$_2$O$_2$ and S-nitrosoalbumin. Furthermore, the enhanced inhibition was observed both for GSNO and NO in the presence of plasma, although higher concentrations of both were required to inhibit aggregation in platelet-rich plasma compared with their actions on WP. Nevertheless, in the presence of H$_2$O$_2$, GSNO inhibited platelet activation in PRP at concentrations below 30 nM. In addition, H$_2$O$_2$ alone was able to inhibit platelet activation, presumably through interactions with endogenous NO donors, since WP activated by thrombin did not respond to H$_2$O$_2$ in this way. Inhibition of the breakdown of H$_2$O$_2$ using a glutathione peroxidase inhibitor enhanced the inhibitory effects of H$_2$O$_2$. Glutathione peroxidase has been reported to be inhibited by NO [28].

Free nitrosothiols were reported to occur in plasma at concentrations of 1–3 µM [3] in addition to the presence of a micromolar concentration of nitrosated proteins. These concentrations are considered to be high, since the availability of NO would have large effects on vasomotor tone. It is now well established that transnitrosation also takes place between free amino acids with thiol residues and thiols on proteins [29], although the rate of transfer of NO from nitrosoalbumin to cysteine is slow, approximately 1%/min [30]. Some plasma nitrosothiols may be more labile than GSNO and release their NO even more readily or transfer NO to the pools of glutathione within the platelet. S-Nitrosoalbumin showed a potent inhibitory effect on platelets, greater than observed previously by others [3], S-nitrosocysteine is also a potent platelet inhibitor [11]. Glutathione peroxidase, which is very active in platelets, has been shown to transnitrosate between GSNO and albumin, but not from S-nitrosocysteine [31]. The evidence presented above clearly shows that inhibition by nitrosothiols, including S-nitrosoalbumin, is strongly enhanced by the presence of H$_2$O$_2$. Therefore both plasma free and protein-bound nitrosothiols, as well as NO released from the endothelium, may have a physiological role in the inhibition of platelets, since they are each active at very low concentrations, particularly in the presence of H$_2$O$_2$.

It has been reported previously that H$_2$O$_2$ inhibits platelet activation [17,32] or S-hydroxytryptamine release [33] in WP and in PRP, but at concentrations of peroxide much higher than those used in these studies. Ambrosio et al. [34] found that generation of exogenous reactive oxygen species by xanthine/xanthine oxidase inhibited platelet activation, but that this was reversed by catalase and not superoxide dismutase. We have now shown that these inhibitory effects of H$_2$O$_2$ are mediated by guanylate cyclase, since two distinct inhibitors of this enzyme abolish the effects of H$_2$O$_2$ in PRP. This is in agreement with the previous study [34] and suggests that the peroxide is likely to be acting through endogenous NO donors in the plasma or platelets. The contribution of NO synthesized endogenously in the platelets appears to be small, since 1-NAME reduced the inhibition only slightly. H$_2$O$_2$ alone inhibited platelet aggregation in plasma by 26 % and, applying this to Figure 3, right panel, it is possible to infer that the total nitrosothiol concentration may be in the region of 15–25 nM. Exposure of platelets to NO may arise from release of NO by other platelets, from endothelial cells or from plasma nitrosothiols. H$_2$O$_2$ may be released from platelets during activation or through the activity of superoxide dismutase on endothelial cells. NO or nitrosothiols together with H$_2$O$_2$ are powerful inhibitors of platelet activation at very low concentrations in the plasma.

Advances were made in the understanding of the nature of this synergism. Pre-mixing the GSNO with H$_2$O$_2$ before addition to the platelets had little influence on the observed synergy in their
inhibitory effects. Furthermore, $\text{H}_2\text{O}_2$ did not increase significantly the rate of release of NO from GSNO nor enhance the degradation of NO, indicating there was no direct interaction between these two mediators. The time interval between the addition of GSNO and $\text{H}_2\text{O}_2$ was not critical, but tended to be optimal when added together, suggesting some flexibility in the conditions required for this synergism to occur. The addition of authentic NO and $\text{H}_2\text{O}_2$ was more sensitive to the separation in time, but the synergism was still strong even if the additions are separated for up to 1 min, whether the $\text{H}_2\text{O}_2$ is added before the NO or vice versa. These observations may give important clues to the location of the interaction of the NO and $\text{H}_2\text{O}_2$ and appear to add further evidence against the formation of a separate inhibitory species resulting from a prior reaction between NO and $\text{H}_2\text{O}_2$. A possible effect of $\text{H}_2\text{O}_2$ through its known enhancement of cyclo-oxygenase activity was excluded when identical interactions with GSNO were found in aspirin-treated platelets.

The fact that $\text{H}_2\text{O}_2$ may be added after NO and still act synergistically indicates that NO binds first to the haem group of guanylate cyclase to form nitrosohaem, but at concentrations below those required to activate the enzyme in the absence of $\text{H}_2\text{O}_2$. Either the affinity of the haem for the residual NO is increased by $\text{H}_2\text{O}_2$, or, more likely, its action is increased by the peroxide after binding of the NO. NO has a very rapid action on platelets, activating guanylate cyclase and increasing the concentration of cGMP within a few seconds, reaching a plateau (in the presence of isobutylmethylxanthine) that endures for some minutes [35,36]. In previous work [20], the effects of simultaneous addition of NO and $\text{H}_2\text{O}_2$ were accounted for, to a significant extent, by the enhancement of the activation of guanylate cyclase. In this study, there was some variation in guanylate cyclase activity, depending on the time at which the $\text{H}_2\text{O}_2$ was added to the platelets relative to the NO. The synthesis of cGMP paralleled the variation in inhibitory activity of the NO/$\text{H}_2\text{O}_2$ additions at the equivalent time intervals. In addition, the guanylate cyclase inhibitors LY88385 and ODQ abolished the inhibition of aggregation by both GSNO and GSNO/$\text{H}_2\text{O}_2$, again suggesting the phenomenon is strongly dependent on cGMP. There was slight variation in the effectiveness of the inhibitors, which may relate to differences in their mechanism of action. LY88385 is thought to inhibit by generation of superoxide anions that neutralize NO, not a true inhibitor [37], whereas ODQ directly inhibits the enzyme [38,39]. These results indicate that guanylate cyclase activity is essential to the effects of $\text{H}_2\text{O}_2$.

Exogenous cGMP (dBcGMP) exhibited interactions with $\text{H}_2\text{O}_2$ that were much smaller than those for GSNO or NO, suggesting that the primary action of $\text{H}_2\text{O}_2$ occurs before the formation of cGMP. In contrast, large increases in the synthesis of cGMP were seen with NO in the presence of $\text{H}_2\text{O}_2$. However, it was reported [40] that $\text{H}_2\text{O}_2$ had no direct effect on soluble guanylate cyclase activity, an observation supported by our own work [20]. NO is thought to cause a relocalization of the haem iron away from the porphyrin ring, which permits a change in the conformation of the enzyme protein, thereby increasing its activity [41]. $\text{H}_2\text{O}_2$ increases the oxidation state of haemoproteins to III or higher ferri forms (IV,V) [42,43] and, in large excess, may lead to the complete delocalization of the iron from these proteins [44]. Fe(III) is less able to bind NO and, therefore, does not appear to offer an obvious mechanism for the enhancement of guanylate cyclase activity. Nevertheless, it is possible that the synergy of NO and $\text{H}_2\text{O}_2$ may occur at this level: such interactions were shown in relation to the oxidation states of myoglobin haem where NO lowers the haem from oxidation states IV and V to III [45], but further investigations would be required to establish this. $\text{H}_2\text{O}_2$ alone cannot activate guanylate cyclase, but, in the presence of catalase, another haemoprotein, activation of the enzyme may occur [46]. In addition, there is evidence that the generation of hydroxyl radicals, which are reported to be activators of guanylate cyclase [47], may contribute to the synergism of NO and $\text{H}_2\text{O}_2$, since this was inhibited by hydroxyl-radical scavengers: the scavengers have no effect on the activation of the enzyme by NO alone. Soluble guanylate cyclase from human platelets has been shown to be activated reversibly by synergism between NO donors and low concentrations of oxidizing agents such as diamine and disulphide 4,4′-dithiopyridine, which was associated with the formation of partial disulphide bridges [48]. An excess of these agents inhibited the enzyme activity. Therefore it was proposed that mild oxidative stress may enhance the activation of guanylate cyclase and make it more sensitive to low concentrations of NO. A role for $\text{H}_2\text{O}_2$ in cell signalling has been proposed previously, but not in respect to guanylate cyclase [49].

In conclusion, it has been demonstrated that $\text{H}_2\text{O}_2$ would strongly enhance the inhibition of platelet aggregation induced by a stable nitrosothiol and that these events occur within the platelet. The mechanism of this interaction is likely to involve the activation of guanylate cyclase. It may involve the generation of other radical species or a change in the conformation of the haem or protein to make the enzyme more sensitive to NO. These effects of $\text{H}_2\text{O}_2$ may not be exclusive to platelets, since it has also been shown to potentiate other NO-related events, including pulmonary vasodilatation [14] and arterial endothelium-dependent relaxation [15]. Much remains to be learned about the mechanism.

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