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 H_2O_2 . In the present study, the interactions of H_2O_2 with nitrosothiols, the influence of the presence of plasma and the mechanism of the synergism were investigated. H_2O_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 H_2O_2 . The synergism with H_2O_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 H_2O_2 was completely inhibited by guanylate cyclase inhibitors. Synergism was also observed whether the H_2O_2 was added simultaneously or 1 min before or after the GSNO (or NO). This suggests that the action of H_2O_2 follows the occupation by NO of haem sites in guanylate cyclase and that a prior

reaction between NO and H_2O_2 was not required. In the absence of exogenous GSNO or NO, H_2O_2 inhibited activation of platelets in plasma, an effect abolished by guanylate cyclase inhibitors. This suggested that endogenous NO donors in plasma or NO synthesized in platelets may interact with H_2O_2 . Addition of N^G -nitro-L-arginine methyl ester (hydrochloride) (L-NAME) decreased the effects of the H_2O_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 H_2O_2 was also enhanced by β -mercaptosuccinate, a glutathione peroxidase inhibitor that protects the H_2O_2 . These results suggest a potent synergism of H_2O_2 with endogenous plasma nitrosothiols that inhibit platelet function through an intracellular mechanism involving guanylate cyclase.

INTRODUCTION

NO is synthesized from 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 nitrites 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 *in vitro* [10,11] and *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.

H_2O_2 is produced by dismutation of superoxide anion ($O_2^{\cdot -}$) [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]. H_2O_2 , in the absence of other agonists, induces primary aggregation of washed plate-

lets (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 H_2O_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 H_2O_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 H_2O_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 H_2O_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 H_2O_2 are added simultaneously to the platelets. Further information is required on the mechanism of this synergism.

MATERIALS AND METHODS

H_2O_2 , sodium urate, prostacyclin (synthetic sodium salt), thrombin (human), glutathione, N^G -nitro-L-arginine methyl ester (hydrochloride) (L-NAME), dibutyl cGMP (dBcGMP), isobutyl-

Abbreviations used: GSNO, S-nitrosoglutathione; DTPA, diethylenetriaminepenta-acetic acid; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide; dBcGMP, dibutyl cGMP; IC_{50} , concentration required to produce inhibition of 50 %; LY85835, 6-anilino-5,8-quinolinequinone; ODQ, oxadiazoloquinoline-1-one; L-NAME, N^G -nitro-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-anilino-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 NaH_2PO_4 , 7 mM NaHCO_3 , 2.7 mM KCl, 0.5 mM MgCl_2 , 5.6 mM glucose, pH 7.4) and diluted to a count of 3×10^8 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*-nitrosoalbumin and H_2O_2 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*-Nitrosoalbumin was prepared by incubation of human serum albumin with acidified sodium nitrite, and the degree of nitrosation was determined using the Griess reaction [23]. H_2O_2 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 H_2O_2 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 $\mu\text{g}/\text{ml}$ final concentration) or thrombin (0.02 unit/ml) and measured as the increase in light transmittance after 3 min. NO, GSNO or *S*-nitrosoalbumin, either in combination with H_2O_2 (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 H_2O_2

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 Hepes 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 H_2O_2 .

Radioimmunoassay for cGMP

The platelets were treated with agonists and antagonists at the appropriate interval of time, in the presence of isobutylmethylxanthine (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 $\pm$ S.E.M. of three to five independent experiments unless stated otherwise. Statistical evaluation was by unpaired Student's *t* test. The IC_{50} 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 (IC_{50} 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 IC_{50} 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 Inhibition of platelets by NO and GSNO: influence of carboxy-PTIO

Carboxy-PTIO (1 μM) was incubated with WP at 37 °C with continual stirring for 1 min before the addition of NO or GSNO (0.001–1 μM). After a further 1 min incubation, thrombin (0.02 unit/ml) was added and the extent of platelet aggregation was measured 3 min later. The effect of carboxy-PTIO on NO is represented by (○), compared with NO (●) or GSNO (■) where buffer was added instead of carboxy-PTIO. The results are expressed as percentages of inhibition of platelet aggregation and represent the means $\pm$ S.E.M. of four independent experiments.

Figure 2 Enhancement by H_2O_2 of the inhibition of platelet aggregation induced by GSNO

Top: the effects of H_2O_2 on inhibition of platelets by a range of concentrations of GSNO. WP were pre-incubated with GSNO (0–1 μM) before the addition of thrombin (0.02 unit/ml) either in the presence (○) or absence (●) of H_2O_2 , with aggregation measured 3 min after the addition of thrombin; a mixture of glutathione (100 nM) and H_2O_2 was a control (▲). The influence of carboxy-PTIO (1 μM) is also shown (△). The H_2O_2 was added simultaneously with the GSNO. The data are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of five individual experiments. Middle: the effects of a range of concentrations of H_2O_2 on inhibition of platelets by GSNO. GSNO (10 μM) was added simultaneously with H_2O_2 (0–20 μM ; □) to WP and incubated for 1 min before the addition of thrombin (0.02 unit/ml); the extent of aggregation was measured after 3 min. In a control experiments, H_2O_2 that had been exposed to air overnight with continual stirring (■) was added with GSNO. The results are the means $\pm$ S.E.M. of four experiments. Bottom: the effects of H_2O_2 on the inhibition of platelets by S-nitrosoalbumin. WP were pre-incubated with S-nitrosoalbumin (0–1 μM) before the addition of thrombin (0.02 unit/ml) either in the presence (○) or absence (●) of H_2O_2 ; aggregation was measured 3 min after the addition of thrombin. H_2O_2 was added simultaneously with S-nitrosoalbumin. The data are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of three individual experiments.

(100 nM) inhibited aggregation by $52.5 \pm 2.4\%$, but in the presence of carboxy-PTIO (1 μM) this was reduced to $2.5 \pm 2.2\%$. In contrast, carboxy-PTIO at this concentration did not affect platelet inhibition induced by GSNO. The IC_{50} 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 H_2O_2 to WP

H_2O_2 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 H_2O_2 (20 μM) resulted in a concentration-dependent inhibition of thrombin-induced aggregation: the IC_{50} for GSNO in the presence of H_2O_2 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 H_2O_2 ($P < 0.05$) (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 H_2O_2 , 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 H_2O_2 was effective, the experiments were repeated with a fixed concentration of GSNO (10 nM) and H_2O_2 from 0.01–20 μM (Figure 2, middle). The EC_{50} for H_2O_2 was 2.7 ± 0.3 μM , and inhibition was maximal at 20 μM ($96.2 \pm 2.1\%$). The term EC_{50} (effective concentration at 50%) is used to describe the actions of H_2O_2 , since it increased the action of GSNO. Enhancement of GSNO-mediated inhibition was not observed with H_2O_2 solutions that had been either left exposed overnight or heated at 100 °C for 30 min (Figure 2, middle).

Pre-mixing GSNO and H_2O_2 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 $24.8 \pm 6.2\%$ 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 H_2O_2 (20 μM) added simultaneously and directly to platelets induced $94.2 \pm 4.3\%$ inhibition, and again this was unchanged if the reagent were pre-mixed for 45 s before addition to the platelets. Premixing for 1 min led to a small reduction in the level of inhibition ($86.8 \pm 3.6\%$, $n = 3$), 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 (IC_{50} 70 ± 18 nM) (Figure 2, bottom). Furthermore, the S-nitrosoalbumin also acted synergistically with 20 μM H_2O_2 , which decreased significantly the IC_{50} to 15 ± 7.5 nM ($P < 0.05$, $n = 4$).

It has been reported that H_2O_2 enhances the activity of cyclooxygenase in a number of cell types; therefore the synergism of GSNO and H_2O_2 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 H_2O_2 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 H_2O_2 , as was observed previously with authentic NO and H_2O_2 [20].

Figure 3 Influence of NO or GSNO and H₂O₂ on platelet aggregation in the presence of plasma

Left: PRP was incubated with NO (0.001–10 μ M) in the presence (●) or absence (○) of H₂O₂ for 1 min before the addition of collagen (0.5 μ g/ml), and aggregation was measured 3 min later. In all cases the H₂O₂ was added to the platelets simultaneously with NO. The results are expressed as the percentages of inhibition of platelet aggregation and represent the means $\pm$ 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 H₂O₂ (○).

Table 1 Effects of glutathione peroxidase inhibitors on platelet inhibition by H₂O₂ in the presence of plasma

β -mercaptosuccinate (10 μ M) or *N*-ethylmaleimide (5 μ M) was incubated with WP at 37 °C with continual stirring for 1 min before the addition of H₂O₂ (20 μ M). After a further 1 min incubation, collagen (0.5 μ g/ml) was added, and aggregation was measured 3 min later. The results are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$.

Addition	H ₂ O ₂ (μ M)	Inhibition (%)
None	20	21 $\pm$ 4.5
β -Mercaptosuccinate	20	41 $\pm$ 7.5*
<i>N</i> -Ethylmaleimide	20	43 $\pm$ 9*

Table 2 Effect of an NO synthase inhibitor on platelet inhibition by H₂O₂ in the presence of plasma

L-NAME (100 μ M) was incubated with WP at 37 °C with continual stirring for 5 min before the addition of H₂O₂ (1–20 μ M). After a further 1 min incubation, collagen (0.5 μ g/ml) was added, and aggregation was measured 3 min later. The results are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$.

Addition	H ₂ O ₂ (μ M)	Inhibition (%)
None	1	0
	5	4 $\pm$ 2.1
	10	20 $\pm$ 2.2
	20	36 $\pm$ 3.7
L-NAME (100 μ M)	1	0
	5	2.5 $\pm$ 1.7
	10	12 $\pm$ 2.1*
	20	24 $\pm$ 4*

The influence of H₂O₂ 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 μ M) or GSNO (0.01–10 μ M), although greater concentrations of these inhibitors were required than those for WP. The IC₅₀ values were 826 $\pm$ 168 nM and 477 $\pm$ 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 μ M) and H₂O₂ (1–500 μ M) inhibited aggregation stimulated by collagen to a greater extent than NO alone, the IC₅₀ falling to 149 $\pm$ 66 nM (Figure 3, left panel). The synergism between GSNO and H₂O₂ was also observed in PRP. GSNO (0.01–10 μ M) and H₂O₂ added simultaneously to PRP inhibited aggregation induced by collagen to a greater extent than GSNO alone (Figure 3, right panel). The IC₅₀ for GSNO in the presence of H₂O₂ (20 μ M) was reduced 15-fold from 477 $\pm$ 162 to 32 $\pm$ 9 nM ($P < 0.05$). The addition of *S*-nitrosoalbumin (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 H₂O₂ (results not shown).

If H₂O₂ 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 μ M or above (Tables 1 and 2). This did not occur in WP, suggesting that the H₂O₂ was acting synergistically with endogenous NO

donors. The inhibitory action of H₂O₂ was enhanced by addition of β -mercaptosuccinate (10 μ M), 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 μ M) for 5 min to inhibit platelet NO synthesis, then the effects of H₂O₂ 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 μ M L-arginine to cause near-maximal inhibition of NO synthase after 3 min [26]. At higher concentrations of L-NAME (800 μ M) and with longer pre-incubation times with this inhibitor (15 min), no greater reduction in the H₂O₂-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 H₂O₂ can also play a role in the synergism (results not shown) and indicating a role for the platelet glutathione peroxidase in regulating platelet H₂O₂ release.

Figure 4 Effects of separating the additions of NO or GSNO and H₂O₂ on the inhibition of platelet aggregation

Left: H₂O₂ (20 μ M) was added to WP either simultaneously or up to 1 min before or after NO (●) or GSNO (○) (each 10 nM). In all cases a 1 min gap between addition of NO/GSNO and that of thrombin (0.02 unit/ml) was maintained, so in some experiments the H₂O₂ was added immediately before the thrombin. Aggregation was measured 3 min after the addition of the thrombin. For comparison, the inhibition induced by NO (10 nM) alone is also shown (▲). The results are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of three independent experiments. Right: the experiments were performed as for the left panel, except that the platelets were preincubated with isobutylmethylxanthine. Excess unlabelled HCl was added after 1 min instead of thrombin, and the accumulated cGMP content was measured by radioimmunoassay. The influence of NO (10 nM) alone on platelet cGMP concentrations is illustrated for comparison (▲).

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 $92.5 \pm 1.1\%$ (buffer control) to $75.9 \pm 4\%$ for urate ($P < 0.05$, $n = 3$) and to $80 \pm 3.3\%$ ($P < 0.05$, $n = 3$) 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 fate 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 μ M) was added up to 1 min before or after addition of GSNO or NO (both 10 nM). Simultaneous addition of GSNO/H₂O₂ induced $92.2 \pm 2.1\%$ 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 $61.1 \pm 6\%$ ($P < 0.01$ compared with the simultaneous addition of the reagents), whereas incubation for 30 s beforehand induced an inhibition of $72.2 \pm 1.1\%$ ($P < 0.01$). 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 μ M 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 $90 \pm 3\%$ (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

Figure 5 Influence of H₂O₂ on inhibition of platelets by dBcGMP

WP were incubated with a range of dBcGMP concentrations for 1 min before the addition of thrombin (0.02 unit/ml). The experiments were performed with dBcGMP (0–10 mM) alone (●) or with H₂O₂ (20 μ M) added simultaneously with the dBcGMP (□). Aggregation was measured 3 min after the addition of the agonist. The results are expressed as percentages of inhibition of aggregation and represent the means $\pm$ S.E.M. of three independent experiments.

addition, the inhibitory actions of H₂O₂ 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 H₂O₂ 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 IC₅₀ for dBcGMP was 220 ± 30 μ M, and complete inhibition was achieved at 2 mM. Addition of H₂O₂ (25 μ M) in combination with dBcGMP (0–5 mM) led to an enhancement of the inhibitory actions of dBcGMP. The IC₅₀ for dBcGMP in the presence of H₂O₂ was reduced to 100 ± 10 μ M ($P < 0.05$), whereas complete inhibition was unchanged at 2 mM. The magnitude of the change was relatively modest in comparison with the effects of H₂O₂ on the action of NO and GSNO.

As indicated above, the interaction of NO and H₂O₂ 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 H₂O₂, was paralleled to a significant degree by variations in the concentrations of cGMP (Figure 4, right panel), providing further evidence of the importance of the role of guanylate cyclase in the interactions of H₂O₂ and NO.

DISCUSSION

GSNO has a biological half-life on platelets several times longer than authentic NO [22]. The synthesis of both NO and H₂O₂ 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 H₂O₂, but to a slightly lesser extent than reported earlier for authentic NO and this peroxide [20]. This synergism occurred in WP at very low concentrations of GSNO (less than 1 nM) and at physiologically relevant concentrations of H₂O₂. The interaction of GSNO and H₂O₂ was not inhibited by an excess of carboxy-PTIO (unlike the interactions of NO and H₂O₂), indicating that these interactions

occur intracellularly. Similar interactions on the inhibition of platelet aggregation were also observed between H₂O₂ 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 H₂O₂, GSNO inhibited platelet activation in PRP at concentrations below 30 nM. In addition, H₂O₂ alone was able to inhibit platelet activation, presumably through interactions with endogenous NO donors, since WP activated by thrombin did not respond to H₂O₂ in this way. Inhibition of the breakdown of H₂O₂ using a glutathione peroxidase inhibitor enhanced the inhibitory effects of H₂O₂. Glutathione peroxidase has been reported to be inhibited by NO [28].

Free nitrosothiols were reported to occur in plasma at concentrations of 1–3 μ M [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 H₂O₂. 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 H₂O₂.

It has been reported previously that H₂O₂ inhibits platelet activation [17,32] or 5-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 H₂O₂ are mediated by guanylate cyclase, since two distinct inhibitors of this enzyme abolish the effects of H₂O₂ 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 L-NAME reduced the inhibition only slightly. H₂O₂ 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. H₂O₂ may be released from platelets during activation or through the activity of superoxide dismutase on endothelial cells. NO or nitrosothiols together with H₂O₂ 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 H₂O₂ before addition to the platelets had little influence on the observed synergism in their

inhibitory effects. Furthermore, H_2O_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 H_2O_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 H_2O_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 H_2O_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 H_2O_2 and appear to add further evidence against the formation of a separate inhibitory species resulting from a prior reaction between NO and H_2O_2 . A possible effect of H_2O_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 H_2O_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 H_2O_2 . Either the affinity of the haem for the residual NO is increased by H_2O_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 H_2O_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 H_2O_2 was added to the platelets relative to the NO. The synthesis of cGMP paralleled the variation in inhibitory activity of the NO/ H_2O_2 additions at the equivalent time intervals. In addition, the guanylate cyclase inhibitors LY83835 and ODC abolished the inhibition of aggregation by both GSNO and GSNO/ H_2O_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. LY85835 is thought to inhibit by generation of superoxide anions that neutralize NO, not a true inhibitor [37], whereas ODC directly inhibits the enzyme [38,39]. These results indicate that guanylate cyclase activity is essential to the effects of H_2O_2 .

Exogenous cGMP (dBcGMP) exhibited interactions with H_2O_2 that were much smaller than those for GSNO or NO, suggesting that the primary action of H_2O_2 occurs before the formation of cGMP. In contrast, large increases in the synthesis of cGMP were seen with NO in the presence of H_2O_2 . However, it was reported [40] that H_2O_2 had no direct effect on soluble guanylate cyclase activity, an observation supported by our own work [20]. NO is thought to cause a relocation 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]. H_2O_2 increases the oxidation state of haemoproteins to III or higher ferryl 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 H_2O_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. H_2O_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 H_2O_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 H_2O_2 in cell signalling has been proposed previously, but not in respect to guanylate cyclase [49].

In conclusion, it has been demonstrated that H_2O_2 will 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 H_2O_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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