Hydrogen peroxide at low concentrations strongly enhances the inhibitory effect of nitric oxide on platelets

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Simultaneous application of NO and H₂O₂ to human platelets at physiologically relevant concentrations increased inhibition of platelet aggregation by NO almost 100-fold. If NO and H₂O₂ were mixed before the addition to platelets, the inhibitory effect remained but still depended on the presence of NO. This suggested an enhanced sensitivity of the platelets to residual NO

in the presence of H₂O₂. The inhibition by the NO/H₂O₂ mixture was not due to peroxynitrite and was only partly reversed by radical scavengers. The mechanism includes enhanced formation of cyclic GMP in response to NO in the presence of H₂O₂. H₂O₂ may play a positive physiological role by amplification and/or prolongation of the action of NO.

INTRODUCTION

Platelet activation is inhibited by NO [1] which is biosynthesized from L-arginine in vascular endothelium by NO synthase [2]. NO binds to the haem of platelet guanylate cyclase, stimulating formation of cyclic GMP [3] which inhibits platelet activation. Platelets also biosynthesize NO from L-arginine when activated by some agonists [4]. NO may thus possess antithrombotic properties of physiological importance as well being a vasodilator. H₂O₂ is formed in the endothelium through reduction of O₂^{•-} anions by superoxide dismutase. O₂^{•-} anions are known to form peroxynitrite with NO [5], but the interaction of NO with H₂O₂ is less clear. Peroxynitrite has recently been reported to show both stimulatory and inhibitory effects on platelet aggregation [6]. H₂O₂ alone is a weak activator of platelets [7] and, like NO, may be formed when platelets were activated [8]. There is evidence that lipid peroxides (or oxidized lipoproteins) also cause a greater sensitivity of platelets to agonists [9,10], but nothing is known of their effects on inhibition of platelets by NO. Organic peroxy radicals and peroxides form adducts with NO, but there is no indication that this is of physiological relevance [11,12]. The effects of combinations of NO and H₂O₂ on platelet function were investigated in this study and the results indicate that NO and H₂O₂ may interact synergistically to inhibit platelet activation.

EXPERIMENTAL

Materials

Superoxide dismutase (bovine), D-mannitol, L-ascorbate, H₂O₂, sodium urate, prostacyclin (prostaglandin I₂; sodium salt) and thrombin (human) were all purchased from Sigma, Poole Dorset, U.K. Collagen (type I) in solution (equine) was from Hormonchemie G.m.b.H., Munich, Germany. NO gas was from Lynde Gas, Stoke on Trent, Staffs., U.K., and the cyclic GMP radioimmunoassay kit from Amersham International, Amersham, Bucks., U.K. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (Carboxy-PTIO) was purchased from Calbiochem-Novabiochem (U.K.), Nottingham, Notts., U.K.

Isolation of platelets

Venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the preceding 14

days. Blood was taken into acid/citrate/dextrose anticoagulant and centrifuged for 20 min at 150 *g* to yield platelet-rich plasma. The platelet-rich plasma was re-centrifuged in the presence of 50 nM prostacyclin at 800 *g* for 10 min to obtain a platelet pellet [13]. This was suspended in physiological buffer (NaCl 150 mM, Hepes 5 mM, NaH₂PO₄ 0.55 mM, NaHCO₃ 7 mM, KCl 2.7 mM, MgCl₂ 0.5 mM, glucose 5.6 mM, pH 7.4) and diluted to a count of 3 × 10⁸ platelets/ml. The isolated platelets were left for 1 h at room temperature to recover their sensitivity to aggregating agents. In other experiments, the second centrifugation step was repeated after pretreatment of platelets with 25 μM H₂O₂ or buffer and without further addition of prostacyclin and resuspended in buffer. An alternative method was also used to isolate platelets by lowering the pH during centrifugation [14]. In other experiments the Hepes buffer was replaced with a modified Tyrode's buffer (NaCl 137 mM, NaHCO₃ 11.9 mM, NaH₂PO₄ 4.2 mM, KCl 2.7 mM) for all the procedures of platelet isolation and experimentation.

NO and H₂O₂ solutions

NO was prepared as solutions with a range of concentrations [15]. Double-distilled water was boiled for 15 min, allowed to cool and drawn under vacuum into gas sampling tubes. The water was bubbled with N₂ for 1 h and sealed with a rubber septum. The appropriate amount of NO was then injected into the deoxygenated water via gas-tight syringes. Controls were prepared by exposing the NO solutions to air for 15 min. The concentrations of the stock NO solutions were checked with the Griess reaction [16] and with an NO electrode (Diamond Electro-Tech, Ann Arbor, MI, U.S.A.) calibrated against NO/argon gas standards.

H₂O₂ was diluted with deoxygenated water from a stock solution (8.82 M) and kept sealed in air-tight vials throughout the experiments. Controls were prepared by exposing the H₂O₂ to air overnight before dilution.

In some experiments, H₂O₂ and NO solutions were pre-mixed in the platelet buffer (100 μl) at concentrations 20 times the final concentration, i.e. the same concentrations as used for the equivalent stock concentrations of NO and H₂O₂ in the previous experiments. Mixing was carried out in a separate platelet aggregometer microcuvette (with exposure to air) at 37 °C, with

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide.

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stirring at 1000 rev./min; 5 μ l aliquots were removed 10–60 s after mixing and transferred (in 2–3 s) to a second microcuvette containing 100 μ l suspensions of platelets.

Preparation of peroxyntirite

Sodium peroxyntirite was prepared as described previously [17]; controls were prepared by the same procedure except that the solutions were passed into water instead of NaOH, leading to immediate decomposition of the oxidant. NaOH was added later to produce the same pH. The concentration of stock peroxyntirite preparation was determined spectrophotometrically ($\epsilon_{302\text{nm}} = 1670 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using decomposed peroxyntirite as the blank. All solutions were kept on ice before mixing and the vessel containing NaOH was kept on ice throughout the preparation.

Platelet-aggregation tests

Isolated platelet suspensions (100 μ l) were incubated in 300 μ l microcuvettes for 1 min at 37 °C with continual stirring at 1000 rev/min (magnetic stir bar) in a Payton dual-channel aggregometer (Centronic Sales, Croydon, Surrey, U.K.) before the addition of agonists or antagonists. Platelet aggregation was induced by the addition of thrombin (0.02 unit/ml), collagen (0.5 μ g/ml) or H_2O_2 (0–25 μ M) (final concentrations) and measured as increases in light transmission [18] after 3 min. NO or peroxyntirite was added 1 min before the thrombin. NO and H_2O_2 were added from separate syringes simultaneously 1 min before the addition of thrombin, except where stated otherwise. The pH of the platelet suspension was checked for each condition.

Formation of cyclic GMP

Platelets were incubated in the presence of isobutylmethylxanthine (200 μ M) to inhibit phosphodiesterase activity. The incubation was stopped 1 min after the addition of NO or NO/ H_2O_2 mix by the addition of 0.5 M HClO_4 , and the concentration of cyclic GMP measured by radioimmunoassay.

RESULTS AND DISCUSSION

Platelets activated with thrombin (0.02 unit/ml) (Figures 1a, 1c and 1d) or collagen (0.5 μ g/ml) were all inhibited by low concentrations of NO, delivered as a bolus with a microsyringe, with an IC_{50} of 110 ± 30 and 18.0 ± 3 nM respectively for the two agonists. In contrast, H_2O_2 did not inhibit agonist-induced platelet aggregation at any of the concentrations used (not shown) and, as found by others, produced a primary aggregation of the platelets at concentrations above 25 μ M (Figure 1b). Simultaneous injection of NO and H_2O_2 over a range of concentrations strongly increased the inhibition caused by NO (Figures 1e and 1f), so that, at 25 μ M H_2O_2 , NO showed inhibitory activity even at a concentration of 1 nM, an increase in potency of almost two orders of magnitude (Figure 2a). The precise concentration of NO required to cause 100% inhibition in the presence of H_2O_2 varied in preparations from different donors, as is the case for other platelet inhibitors, so that in separate groups of experiments 100% inhibition occurred at concentrations as low as 1 nM NO (Figure 2b), the lowest concentration to which NO could be diluted with any accuracy. The effect of the H_2O_2 was still evident at 1 μ M (Figure 2b); at 10 nM NO, the enhancement of inhibition by H_2O_2 was half-maximal at 1.6 ± 0.5 μ M peroxide and at 4.9 ± 2.3 μ M with 1 nM NO. Very similar results were obtained (1) when platelets were isolated by a different procedure using low-pH buffers instead of

Figure 1 Effect of H_2O_2 on the inhibition of platelet aggregation by NO

Isolated platelets were incubated with (a) thrombin (0.02 unit/ml for 3 min) or (b) 25 μ M H_2O_2 . In (c)–(f) combinations of reagents were added 1 min before thrombin: (c) NO (10 nM); (d) NO (100 nM); (e) NO (10 nM) + H_2O_2 (25 μ M); (f) NO (1 nM) + H_2O_2 (25 μ M); (g) as for (e) but thrombin was added 30 min after the addition of NO + H_2O_2 . The results are typical of at least six independent experiments.

prostacyclin [14], (2) in experiments conducted in modified Tyrode's buffer or (3) if collagen was used to aggregate the platelets (not shown).

The inhibition of platelet aggregation by a bolus of NO (100 nM final concentration) had completely disappeared after 30 min (Figure 3), whereas 10 nM NO produced only a slight inhibition (< 10%) and was not measurable after 5 min. The inhibition by 10 nM NO added simultaneously with 25 μ M H_2O_2 was also reversible (Figure 1g), but there was residual inhibition after 30 min (30% of maximal inhibition), with complete recovery of the responses to thrombin after 1 h (Figure 3). At a lower concentration of H_2O_2 (5 μ M) with 10 nM NO, the inhibition also persisted for 15–30 min and again showed full recovery after 1 h. These findings suggest that the inhibition of platelet aggregation by a combination of NO and H_2O_2 is not due to a cytotoxic effect, but that the presence of H_2O_2 effectively prolongs as well as enhances the inhibition.

The inhibitory effects described above were not obtained with solutions of NO left exposed to air for 15 min before addition of

Figure 2 Dose-response relationship between H₂O₂ and NO on inhibition of platelet aggregation by thrombin

(a) Experiments were conducted essentially as described in Figure 1. Inhibition of thrombin (0.02 unit/ml)-induced platelets over a range of NO concentrations (final concn. 10^{-9} – 10^{-5} M) added simultaneously with 0 (□), 5 (●) or 25 (■) μM H₂O₂ or sodium peroxydinitrite (10^{-6} – 10^{-5} M) in the absence of H₂O₂ (○). Results are expressed as percentage inhibition of the maximum aggregation induced by thrombin alone after 3 min and presented as the mean ± S.E.M. of at least four independent experiments. The curves were constructed using a program based on the least-squares method. ▲, As for ■ except that NO was previously exposed to air for 15 min. Platelets were also pretreated, after isolation, for 1 min with 25 μM H₂O₂, re-centrifuged at 150 g for 20 min and resuspended in buffer after which 10 nM NO and 25 μM H₂O₂ were added simultaneously followed by thrombin (△). (b) Inhibition of thrombin (0.02 unit/ml)-induced platelet aggregation by mixtures of NO + H₂O₂ was measured as in (a) except that only two concentrations of NO were used [1 nM (■) and 10 nM (●)] over a range of H₂O₂ concentrations. □, 10 nM NO with expired H₂O₂ (24 h exposure to air). In other experiments H₂O₂ (15 μM) was added 1 min (◇) or 30 min (◆) before the addition of NO (10 nM) followed by thrombin.

H₂O₂ (Figure 2a), demonstrating that neither nitrite nor nitrate derived from the NO produces the same effect. Equally, if the H₂O₂ solution was left unstoppered overnight the interaction with NO disappeared (Figure 2b), indicating that the effects were not due to contaminants in the H₂O₂ solutions. There remains the possibility that the observed effects of H₂O₂ may be due to its interaction with a component of the isolated platelet suspension, e.g. contaminating traces of oxyhaemoglobin, that can inactivate NO, but is itself inactivated by H₂O₂. When platelets were pretreated immediately after isolation with 25 μM H₂O₂ for 1 min, re-centrifuged at 150 g, resuspended in buffer and allowed to recover for 1 h, they showed the same response to NO (IC₅₀ 122 ± 13 nM) as those prepared similarly but pretreated with buffer only (IC₅₀ 109 ± 16 nM). The response to 10 nM NO in the presence of 25 μM H₂O₂ was also the same (Figure 2a). In separate experiments, if 15 μM H₂O₂ was added to platelets 1 min before NO, the inhibitory effect was less by 50%, and disappeared if the H₂O₂ was added 30 min before (Figure 2b).

Figure 3 Recovery of platelets from inhibition by NO/H₂O₂

Experiments were conducted as in Figure 2(a) except that NO alone, at two final concentrations of 10 nM (■) or 100 nM (▲), was added to platelets; 10 nM NO was also added simultaneously with 5 μM (□) or 25 μM (●) H₂O₂. In each case the thrombin (0.02 unit/ml) was added over a range of intervals 1–60 min after the NO. The results are means ± S.E.M. of four independent experiments.

Figure 4 Effect of pre-mixing of NO and H₂O₂ on the inhibition of platelet activation by thrombin

Experiments were conducted as described in Figure 1: thrombin (0.02 unit/ml) was added to the platelets 1 min after the addition of pre-mixtures of NO and H₂O₂ or NO in buffer and the percentage inhibition of aggregation calculated (see Figure 2). All concentrations indicate the final concentrations after addition to platelets. NO and H₂O₂ were pre-mixed in stirred aggregometer cuvettes at concentrations 20 times the final concentrations [NO 10 nM and H₂O₂ 0 (○), 5 (□), 15 (●) and 25 (■) μM] for periods up to 60 s: the time scale does not include the transfer time (2–3 s) and represents the time of pre-mixing. Carboxy-PTIO was added to a concentration tenfold in excess of that of NO 5 s after the mixing of 10 nM NO/25 μM H₂O₂ (▲) or 100 nM NO/buffer (△) and transfer to the platelets after 10 s. The results are means ± S.E.M. of four independent experiments.

These results indicate that the effects of NO are unlikely to be due to an action of H₂O₂ other than one with NO.

One possible explanation is that a molecular species with even greater inhibitory effects than NO is formed by direct interaction between NO and H₂O₂. To test this, NO and H₂O₂ were pre-mixed in a platelet aggregometer cuvette, stirred at 37 °C with different mixtures of the two solutions from 10 to 60 s. Aliquots were transferred to the cuvettes containing the stirred platelets. Transfer between cuvettes took 2–3 s which is in addition to the stated pre-mixing times. After 10 s, the inhibitory activity of NO alone (10 nM) had disappeared (Figure 4), but mixtures of NO (10 nM) and H₂O₂ (5–25 μM) were still fully inhibitory:

Table 1 Effect of reagents that suppress radical oxygen species on the effects of NO and H₂O₂ on platelet activity

Platelets were treated as described in Figure 1 using thrombin as activator, 1 min after the simultaneous addition of NO (10 nM) and H₂O₂ (25 μ M). Maximum aggregation induced by thrombin without NO and H₂O₂ was also determined, and results are expressed as a percentage of this maximum under the conditions described. The results are the means $\pm$ S.E.M. of five independent experiments, except for catalase and DTPA where $n = 2$. The means for two experiments with NO (100 nM) alone are shown.

Addition	Platelet aggregation (%)	
	NO (10 nM) + H ₂ O ₂ (25 μ M)	NO (100 nM)
Control (buffer)	10.8 $\pm$ 3.1	42
+ Sodium urate (0.5 mM)	36.4 $\pm$ 7.0	38
+ Ascorbate (0.2 mM)	9.2 $\pm$ 2.9	30
+ Mannitol (50 mM)	42.8 $\pm$ 6.7	45
+ Superoxide dismutase (100 units/ml)	9.6 $\pm$ 2.9	32
+ Catalase (100 units/ml)	39.0	—
+ DTPA (10 μ M)	12.0	—

inhibition was maintained for longer than 30 s (Figure 4). The question arose whether this inhibitory activity of NO/H₂O₂ at 10 s was due to the continued presence of NO, even at lower concentrations sufficient to inhibit platelets in the presence of H₂O₂. NO is known to be relatively stable in oxygenated water at these low concentrations [19,20] and, over this time scale, losses are only likely to arise as the result of evaporation into the head space of the cuvette which may account for the ultimate fall in inhibitory activity in all the samples. Alternatively, the inhibition may be due to the presence of another species formed on mixing NO and H₂O₂. The NO scavenger carboxy-PTIO [21] was used, in each case in tenfold excess, to sequester NO (it reacts rapidly and stoichiometrically with NO) to investigate what proportion of the inhibitory activity was due to NO itself. Carboxy-PTIO completely reversed inhibition by 100 nM NO when added directly to platelets 1 min before addition of NO (not shown), even when H₂O₂ (final concentration 25 μ M) was added simultaneously with the NO (10 nM final concentration). The scavenger also virtually abolished inhibition (6 $\pm$ 4% remaining) when added 5 s after pre-mixing NO with buffer (no H₂O₂) and 10 s before addition of this NO solution to the platelets (final concentration of NO after addition to platelets 100 nM). When carboxy-PTIO was added to a pre-mixture of NO (final concentration 10 nM) and H₂O₂ (25 μ M), 24 $\pm$ 4% inhibition ($n = 3$) remained, compared with 94 $\pm$ 7% inhibition in the absence of carboxy-PTIO (Figure 4).

NO is clearly of key importance in the enhanced inhibition of platelet aggregation in the presence of H₂O₂, but there remains a further possibility that another reactive species may contribute to the action of NO under these conditions. The effects of scavengers of hydroxyl radicals and singlet oxygen were studied by adding 500 μ M sodium urate, 200 μ M ascorbate or 50 mM mannitol to the platelets (these reagents had no independent effects on platelet aggregation under these conditions): thrombin was added to 0.02 unit/ml 1 min after the addition of 10 nM NO and 25 μ M H₂O₂. Mannitol and urate partly reversed the inhibition by NO/H₂O₂ (Table 1), but neither had any effect on inhibition by NO (100 nM) alone. The addition of bovine blood Cu/Zn superoxide dismutase did not change the inhibition, but human erythrocyte catalase reduced it, presumably by lowering the concentration of H₂O₂ (Table 1). The transition metal ion

Table 2 Effect of NO/H₂O₂ on guanylate cyclase activity in platelets

The formation of cyclic GMP was measured by radioimmunoassay after exposure of isolated platelets for 1 min to NO or H₂O₂ individually or together at the final concentrations indicated. Results are means $\pm$ S.D. of four independent experiments. Statistical analysis was by Student's unpaired *t* test; **P* < 0.05, compared with control; ††*P* < 0.01 compared with NO (0.01 μ M); †*P* = 0.07 compared with NO (0.01 μ M) alone.

Addition	Concentration of NO or H ₂ O ₂ used (μ M)	[Cyclic GMP] (pmol/10 ⁸ platelets)
Control (buffer)		0.39 $\pm$ 0.07
NO	0.01	0.64 $\pm$ 0.06*
	0.10	1.37 $\pm$ 0.22*
	1.00	3.66 $\pm$ 0.50*
H ₂ O ₂	1.00	0.46 $\pm$ 0.09
	5.00	0.67 $\pm$ 0.13
	15.0	0.59 $\pm$ 0.08
	25.0	0.66 $\pm$ 0.19
NO/H ₂ O ₂ [NO (0.01 μ M) constant, H ₂ O ₂ variable]		
	1.0	0.69 $\pm$ 0.11*
	5.0	0.79 $\pm$ 0.09*†
	15.0	1.38 $\pm$ 0.36*††
	25.0	1.45 $\pm$ 0.21*††

chelator diethylenetriaminepenta-acetic acid (DTPA) had no effect on the inhibition of platelet aggregation by the NO/H₂O₂ mixture, suggesting that the effect was not dependent on a metal-mediated formation of radical species from H₂O₂. However, the effects of mannitol and urate indicate that molecular species other than NO may be involved in some aspects of the inhibition by NO/H₂O mixtures.

In order to test whether this inhibition was, at least in part, due to the presence of peroxynitrite, authentic sodium peroxynitrite was freshly prepared and maintained in alkaline conditions. It was diluted immediately before use and its effects on platelets determined: it did not alter the pH of the platelet buffer at the concentrations used. Moro et al. [6] found that higher concentrations (50–150 μ M) of peroxynitrite activate washed platelets. Peroxynitrite inhibited the platelets stimulated by 0.02 unit of thrombin/ml with an IC₅₀ of 3.1 $\pm$ 1.1 μ M (Figure 2a), which is almost 50 times higher (less potent) than NO and 5000 times less potent than the NO/H₂O₂ mix.

The primary mechanism of action of NO is via activation of guanylate cyclase. Cyclic GMP concentrations of platelets were measured after their exposure to low concentrations of NO for 1 min in the presence or absence of H₂O₂, replicating conditions described in the earlier experiments. H₂O₂ alone had no effect on cyclic GMP synthesis. There was some enhancement of NO-induced cyclic GMP synthesis by H₂O₂, but not sufficient to explain all of the phenomena (Table 2). H₂O₂ (25 μ M) in combination with NO (10 nM) induced formation of 1.45 $\pm$ 0.21 pmol of cyclic GMP/10⁸ platelets, comparable with the concentration of cyclic GMP induced by 100 nM NO (1.37 $\pm$ 0.22 pmol/10⁸ platelets); H₂O₂ alone had no significant effect. However, the NO/H₂O₂ mix induced 90–100% inhibition of aggregation whereas NO (100 nM) alone produced only 63.8 $\pm$ 7.6% inhibition.

In conclusion, H₂O₂ enhances the inhibitory action of NO on platelets, but the mechanism is as yet unresolved. These observations contrast with our earlier findings that addition of 25 μ M H₂O₂ to isolated platelets 2 min after a bolus of NO and 1 min after the addition of thrombin decreased the inhibitory action of the NO and increased the IC₅₀ for NO against thrombin-induced aggregation to 3.63 $\pm$ 0.45 μ M [22]. In the latter

experiments the NO may have disappeared after exposure to platelets for 60 s, the H₂O₂ possibly inhibiting the guanylate cyclase which is activated by the NO. This has been observed previously in co-cultures of endothelial and smooth-muscle cells [23], but at much higher concentrations of the peroxide (low millimolar range). H₂O₂ may have other actions on platelet activation or inhibition that influence the effects of NO. In the absence of NO, H₂O₂ acts synergistically with subthreshold concentrations of thrombin or collagen to induce full aggregation, particularly when the peroxide is added after the main agonist [17,22]. This complete contrast in outcome, depending on whether NO is added simultaneously or in advance of the H₂O₂, suggests a special interaction when NO and H₂O₂ are mixed. Addition of H₂O₂ 1 min before the NO also reduces the efficacy of their interaction, but does not eliminate it.

The mechanism and site of this interaction of NO and H₂O₂ require further study. The fact that mannitol and urate inhibit the inhibition by NO, only in the presence of H₂O₂, suggests the involvement of a radical species, e.g. hydroxyl radical or singlet oxygen, at some point in these events, leading to inhibition of the platelets. Hydroxyl radical has been proposed as an activator of soluble guanylate cyclase, whereas H₂O₂ was found to have no direct effect [24]. Hydroxyl radical is generated from H₂O₂ in the presence of transitional metal ions. However, the presence of a metal ion chelator did not reduce the inhibition of platelets by NO in the presence of H₂O₂. Mixtures of NO and H₂O₂ are reported to form singlet oxygen [25], but the duration of the NO/H₂O₂ effects contraindicate a direct role for singlet oxygen. Evidence of a reaction between NO and H₂O₂ producing peroxynitrite [26] at physiological pH is uncertain and there is no evidence for a role for this molecular species in this context.

S-Nitrosoglutathione, an effective inhibitor of platelet aggregation (IC₅₀ 37 nM) which is relatively stable and from which NO is released, probably enzymically [27], has an equally pronounced interaction with H₂O₂ (K. M. Naseem and K. R. Bruckdorfer, unpublished work). This suggests that the important interaction between NO and H₂O₂ may also occur intracellularly and indicates that these observations are not artifacts caused by the release of materials from the isolated platelets. The extracellular formation of another molecular species from NO is unlikely to be the principal event in the interaction between NO and H₂O₂, as indicated by the experiments with carboxy-PTIO, but does not yet preclude the involvement of other species derived from H₂O₂.

The formation of H₂O₂ from O₂^{•-} by endothelial superoxide dismutase may not only be a mechanism for preventing formation of peroxynitrite from NO and O₂^{•-}, but may also have a positive role in the intensification and prolongation of the actions of NO on platelets in the vicinity of the luminal surface of the en-

dothelium. Brain NO synthase is reported to produce H₂O₂ and less NO at low arginine concentrations [28]. This may be a compensatory mechanism to enhance the effect of reduced NO formation. Furthermore, NO and H₂O₂ produced during platelet aggregation may act synergistically to attenuate the effects of some platelet agonists.

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REFERENCES

- Radomski, M. W., Palmer, R. M. J. and Moncada, S. (1987) *Br. J. Pharmacol.* **92**, 181–187
- Palmer, R. M. J., Ashton, D. S. and Moncada, S. (1988) *Nature (London)* **333**, 664–666
- Mellion, B. T., Ignarro, L. J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L. and Kadowitz, P. J. (1981) *Blood* **57**, 946–955
- Radomski, M. W., Palmer, R. M. J. and Moncada, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5193–5197
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620–1624
- Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6702–6706
- Pratico, D., Iuliano, L., Pulcellini, F. M., Bonavita, M. S., Gazzaniga, P. P. and Violi, F. (1992) *J. Lab. Clin. Med.* **119**, 364–370
- Maresca, M., Colao, C. and Leoncini, G. (1992) *Cell. Biochem. Funct.* **10**, 79–85
- Salonen, J. T., Salonen, R., Seppanen, K. et al. (1991) *Am. J. Clin. Nutr.* **53**, 1222–1229
- Meraji S., Moore, C. E., Skinner, V. O. and Bruckdorfer, K. R. (1992) *Platelets* **3**, 155–162
- Padmaja, S. and Huie, R. E. (1993) *Biochem. Biophys. Res. Commun.* **195**, 539–544
- Shelton, J. R. and Kopczewski, R. F. (1967) *J. Am. Chem. Soc.* **32**, 2908–2910
- Radomski, M. W. and Moncada, S. (1983) *Thromb. Res.* **30**, 383–389
- Lagarde, M., Byron, P. A., Guichardant, M. and Dervachanne, M. (1980) *Thromb. Res.* **17**, 581–588
- Palmer, R. M. J., Ferridge, A. G. and Moncada, S. (1987) *Nature (London)* **327**, 524–526
- Griess, J. P. (1879) *Ber. Deutsch. Chem. Ges.* **32**, 426–429
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620–1624
- Born, G. V. R. and Cross, M. J. (1962) *J. Physiol. (London)* **168**, 178–195
- Ford, P. C., Wink, D. A. and Stanbury, D. M. (1993) *FEBS Lett.* **326**, 1–3
- Lancaster, J. R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8137–8141
- Akaike, T., Yoshida, M., Miyamoto, Y. et al. (1993) *Biochemistry* **32**, 827–832
- Naseem, K. and Bruckdorfer, K. R. (1994) *Atherosclerosis* **109**, 187
- Marczin, N., Ryan, U. S. and Catravas, J. D. (1992) *Circ. Res.* **70**, 326–340
- Schmidt, H. H. W. (1992) *FEBS Lett.* **307**, 102–107
- Noronhadutra, A. A., Epperlein, M. M. and Woolf, N. (1993) *FEBS Lett.* **321**, 59–62
- Stamler, J. S., Singel, J. D. and Localzo, J. (1992) *Science* **258**, 1898–1902
- Radomski, M. W., Rees, D. D., Dutra, A. and Moncada, S. (1992) *Br. J. Pharmacol.* **107**, 745–749
- Heizel, B., John, M., Bohme, E. and Mayer, B. (1993) *Biochem. J.* **281**, 627–630