Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta

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1 The effects of hydrogen peroxide (H_2O_2 , 0.1–1 mM) on the tone of the rings of rabbit aorta precontracted with phenylephrine (0.2–0.3 μM) were studied.

2 H_2O_2 induced a concentration-dependent relaxation of both the intact and endothelium-denuded rings. However, in the presence of intact endothelium, H_2O_2 -induced responses were 2–3 fold larger than in its absence, demonstrating the existence of endothelium-independent and endothelium-dependent components of the vasorelaxant action of H_2O_2 .

3 The endothelium-dependent component of H_2O_2 -induced relaxation was prevented by N^G -nitro-L-arginine methyl ester (L-NAME, 30 μM) or N^G -monomethyl-L-arginine (300 μM), inhibitors of nitric oxide synthase (NOS), in a manner that was reversible by L-, but not by D-arginine (2 mM). The inhibitors of NOS did not affect the responses of denuded rings.

4 Methylene blue (10 μM), an inhibitor of soluble guanylate cyclase, blocked H_2O_2 -induced relaxation of both the intact and denuded rings.

5 H_2O_2 (1 mM) enhanced the efflux of cyclic GMP from both the endothelium-intact and denuded rings. The effect of H_2O_2 was 4 fold greater in the presence of intact endothelium and this endothelium-dependent component was abolished after the inhibition of NOS by L-NAME (30 μM).

6 In contrast to the effects of H_2O_2 , the vasorelaxant action of stable organic peroxides, *tert*-butyl hydroperoxide or cumene hydroperoxide, did not have an endothelium-dependent component. Moreover, they did not potentiate the efflux of cyclic GMP from the rings of rabbit aorta.

7 Exogenous donors of NO, specifically, 3-morpholiniosydnonimine (SIN-1), glyceryl trinitrate or sodium nitroprusside were used to decrease the tone of denuded rings to the level induced by endogenous NO released from intact endothelium. This procedure did not influence the vasorelaxant activity of H_2O_2 , showing that H_2O_2 does not potentiate the vasorelaxant action of NO within the smooth muscle.

8 Thus, H_2O_2 -induced relaxation in the rabbit aorta has both endothelium-dependent and independent components. The endothelium-dependent component of the relaxant action of H_2O_2 is due to enhanced endothelial synthesis of NO.

Keywords: Nitric oxide; hydrogen peroxide; endothelium; L-arginine; nitric oxide synthase; nitric oxide synthase inhibitors; methylene blue; polymorphonuclear leukocytes

Introduction

Endothelium-derived relaxing factor (EDRF), a mediator of endothelium-dependent relaxation in the rabbit aorta (Furchgott & Zawadzki, 1980), has recently been identified as nitric oxide (NO) (Palmer *et al.*, 1987). NO also plays a significant role in the cytotoxicity of activated macrophages (reviewed by Nathan & Hibbs, 1991) and is a neuromediator in both the central and peripheral nervous system (reviewed by Snyder & Bredt, 1991). The formation of NO from L-arginine (Palmer *et al.*, 1988a) is catalyzed by a family of FAD and FMN containing haemoproteins (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992) referred to as NO synthases. These enzymes are dioxygenases (Kwon *et al.*, 1990; Leone *et al.*, 1991), the activity of which depends on NADPH and tetrahydrobiopterin (reviewed by Nathan, 1992). Isoforms of NO synthase present in vascular endothelial (Förstermann *et al.*, 1991) and neuronal cells (Bredt & Snyder, 1990) are expressed constitutively and their activity is calcium- and calmodulin-dependent. In contrast, the macrophage isoform is expressed in cells only following their activation by cytokines and bacterial products and

generates NO independently of calcium or calmodulin (Marletta *et al.*, 1988). The activity of NO synthases can be inhibited by N^G -substituted analogues of L-arginine (L-Arg), such as N^G -nitro-L-arginine methyl ester (L-NAME) or N^G -monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988b; Mülsch & Busse, 1990).

The inhibitors of NO synthase cause a pronounced and long lasting elevation of blood pressure and increase the resistance of various vascular beds, indicating that the continuous generation of NO plays a crucial role in the regulation of vascular tone (Rees *et al.*, 1989; Gardiner *et al.*, 1990a,b). Moreover, the release of NO from the endothelium plays an important role in the interactions between the vessel wall and circulating blood cells. Activated platelets release substances such as ADP or 5-hydroxytryptamine which enhance the release of NO from the endothelium (Houston *et al.*, 1985). The released NO relaxes vascular smooth muscle and serves as a negative feedback mechanism, exerting an inhibitory action on the activated platelets resulting in reduced platelet adhesion and aggregation (Radomski *et al.*, 1987a,b).

Several lines of evidence suggest that NO is also an endogenous modulator of certain functions of polymorphonuclear leukocytes (PMNs), including the generation of O_2^- , enzyme release (Schröder *et al.*, 1990) and adhesion to the endothelial cells (Kubes *et al.*, 1991). Conversely, activated PMNs affect the activity of endothelium-derived

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NO. Stimulation of PMNs leads to a rapid increase in the consumption of oxygen and the concomitant release of large amounts of oxygen-derived reactive species including O_2^- and H_2O_2 (Babior *et al.*, 1973). O_2^- shortens the half-life of endothelium-derived NO (Gryglewski *et al.*, 1986). Consistent with these observations are reports showing that activated PMNs induce an endothelium-dependent contraction that can be inhibited by superoxide dismutase (Ohlstein & Nichols, 1989). Less is known about the regulation of vascular tone by H_2O_2 . Early studies investigating the effects of H_2O_2 on the vascular endothelium established that H_2O_2 is responsible for PMN-induced damage of endothelial cells (Weiss *et al.*, 1981). However, at lower than lethal concentrations, H_2O_2 stimulates the biosynthesis of prostaglandins, including vasodilator prostacyclin, by cultured endothelial cells (Harlan & Callahan, 1984; Ager & Gordon, 1984). Finally, H_2O_2 induces the relaxation of bovine pulmonary artery via the stimulation of soluble guanylate cyclase, through a process dependent on the metabolism of H_2O_2 by catalase (Wolin & Burke, 1987).

We investigated the effects of H_2O_2 on the tone of endothelium-intact and denuded rings of rabbit aorta. We have shown that H_2O_2 , at concentrations that do not impair the functional integrity of endothelial cells, induces relaxation in both types of rings; that H_2O_2 -induced relaxation has both endothelium-dependent and independent components and that the endothelium-dependent component of the relaxant action of H_2O_2 is due to the enhanced endothelial synthesis of NO.

Methods

Organ bath experiments

New Zealand rabbit of either sex (2–2.5 kg) were killed with an overdose of pentobarbitone (Verbutal, Biowet, Poland, 50 mg kg⁻¹, i.v.) and their thoracic aortae were isolated and cut into rings of 3 mm width, which were then suspended between stainless steel hooks and mounted in 5 ml organ baths filled with warmed (37°C) and oxygenated (95% O_2 , 5% CO_2) Krebs solution containing 5.6 μ M indomethacin. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 5.6. Denuded rings were prepared by gently rotating the rings on a stainless steel wire prior to mounting. Isometric force was measured with Biegestab K30 type 351 transducers (Hugo Sachs Elektronik, Germany). A tension of 4 g was applied and the rings were equilibrated (60–90 min) by changing the Krebs solution and adjusting the preload to 4 g every 15 min. After a stable baseline was obtained (1.5 h), the rings were contracted with KCl (60 mM) followed by 30 min equilibration in fresh Krebs buffer. The rings were then contracted with phenylephrine at concentrations of 0.2–0.3 μ M, which typically produced 80% of KCl-induced contraction, and acetylcholine (ACh, 0.3 μ M) was added to the baths to test the functional integrity of the endothelium. Only tissues which relaxed by more than 50% of the phenylephrine-induced tone after addition of ACh were considered to have undamaged endothelium. ACh (0.3 μ M) either did not change the tone of endothelium-denuded rings or slight contractions were observed. After replacing the contents of the baths with fresh buffer 5 times every 10 min, the rings were contracted with phenylephrine (0.2–0.3 μ M) and the responses of tissues induced by H_2O_2 (0.1–1 mM) were recorded. L-NAME (30 μ M), L-NMMA (300 μ M) or MeB (10 μ M) were added to the baths 20 min before addition of H_2O_2 or after maximal H_2O_2 -induced relaxation was obtained. Relaxations were expressed as a percentage of the tone induced by phenylephrine. Contractions were expressed as a percentage of the tone induced by KCl (60 mM).

Determination of the efflux of cyclic GMP from the rings of rabbit aorta

Rings of rabbit aorta were obtained as described above. Before the experiments some of the rings were gently rotated on a stainless steel rod to remove their endothelium. The rings were transferred to 1.5 ml Eppendorf tubes containing 0.5 ml of oxygenated Krebs buffer and 0.1 mM isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterases. After 30 min of incubation at 37°C the rings were transferred to another set of Eppendorf tubes containing H_2O_2 , cumene hydroperoxide (CumHP), *tert*-butyl hydroperoxide (*t*-ButHP) (all at 1 mM) or ACh (0.3 μ M) diluted in 0.5 ml of oxygenated Krebs buffer containing 0.1 mM IBMX. The incubation was continued for another 20 min. In some experiments, both the initial and final incubations were carried out in the presence of L-NAME at a concentration of 30 μ M. Following incubation, the rings were removed from the tubes, quickly dried on blotting paper, and weighed. The weights of the rings were typically between 12 and 20 mg. The tubes containing the incubation buffer were stored in a deep freezer (–70°C) until the concentrations of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the buffer were assayed with a specific radioimmunoassay (Amersham, U.K.). The amount of cyclic GMP released from the rings during 20 min of incubation was expressed as fmol mg⁻¹ of tissue weight. The net change of the extravascular release of cyclic GMP by peroxides or ACh was calculated by subtracting the concentrations of cyclic GMP in the presence of tested compounds from the concentration obtained in their absence.

Materials

Indomethacin, acetylcholine HCl (ACh), L-phenylephrine HCl, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), methylene blue (MeB), sodium nitroprusside, catalase (thymol free from bovine liver), superoxide dismutase, isobutylmethylxanthine (IBMX), cumene hydroperoxide (CumHP) and *tert*-butyl hydroperoxide (*t*-ButHP) were purchased from Sigma, H_2O_2 (3% solution) was obtained from a local pharmacy. Glyceryl trinitrate was from Lipha Pharmaceuticals Ltd. (West Drayton, U.K.). SIN-1 (3-morpholiniosydnonimine) was a gift from GEA Ltd. (Copenhagen, Denmark). Lipid peroxidation inhibitor U-83836E ((–)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dichloro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol, dihydrochloride) was a generous gift from The Upjohn Company (Kalamazoo, MI, U.S.A.).

Statistical analysis

Results are expressed as mean $\pm$ s.e. mean of *n* observations. In organ bath experiments, *n* is the number of separate rabbits from which arterial rings used in a particular protocol were obtained. The measurements of the efflux of cyclic GMP were performed in duplicate; consequently, *n* is the number of rings obtained from *n*/2 different rabbits. Statistical differences between means were assessed by two-way unpaired Student's *t* test or, in the case of multiple means, one-way analysis of variance followed by the *post hoc* Bonferroni test or LSD test. In appropriate situations an unpaired two-way one sample *t* test was used. In all cases, a *P* value of 0.05 was considered statistically significant.

Results

Endothelium-dependent and endothelium-independent components of H_2O_2 -induced relaxation

Addition of H_2O_2 (0.1–1 mM) to baths containing intact rings of rabbit aorta precontracted with phenylephrine (0.2–0.3 μ M)

caused a biphasic response consisting of a small, brief contraction followed by a pronounced, sustained relaxation. In rings denuded of endothelium only the relaxant response was observed. As shown in Figure 1, the relaxant effects of H_2O_2 (0.1–1 mM) were concentration-dependent. The removal of endothelium resulted in a 2–3 fold decrease of H_2O_2 -induced relaxation suggesting the existence of endothelium-dependent and endothelium-independent components of H_2O_2 -induced relaxation.

The effect of inhibitors of nitric oxide synthase and methylene blue on H_2O_2 -induced relaxation

Relaxations induced by H_2O_2 (0.75 mM) in intact rings were partially reversed when L-NAME (30 μ M) or L-NMMA (300 μ M) were added to the baths after H_2O_2 -induced relaxation attained its maximum. These inhibitors of NO synthase did not reverse H_2O_2 -induced relaxation in endothelium-denuded rings. In contrast, MeB (10 μ M) reversed the relaxations induced by H_2O_2 in both intact and denuded tissues (Figure 2).

In a separate series of experiments, L-NAME (30 μ M) or L-NMMA (300 μ M) given alone or in combination with L-Arg or D-Arg (both at 2 mM), or MeB (10 μ M) was added 20 min before the addition of H_2O_2 (0.75 mM). Both inhibitors of NO synthase abolished the endothelium-dependent component of

Figure 1 H_2O_2 -induced relaxation of intact and endothelium-denuded rings of rabbit aorta. For experimental details refer to Methods: (a) shows original traces of six rings of rabbit aorta obtained from a single animal. The rings were precontracted with phenylephrine (P, 0.3 μ M). Increasing concentrations of H_2O_2 (0.1–1 mM) elicited a concentration-dependent relaxation both in rings denuded of endothelium (upper 3 traces, E (-)) as well as in those with intact endothelium (lower 3 traces, E (+)). The relaxant responses of the endothelium-intact rings were 2–3 fold larger than those of denuded ones. Addition of H_2O_2 to the baths containing intact rings elicited a biphasic response in which a small, brief contraction was followed by a long, pronounced relaxation. The trace is representative of $n = 7$ identical experiments which are summarized in (b). The responses of rings containing endothelium are depicted as (●), those of denuded rings are shown (○). Points represent means $\pm$ s.e. mean of $n = 7$ different rings.

Figure 2 Reversal by inhibitors of NO synthase and methylene blue of the endothelium-dependent component of H_2O_2 -induced relaxation. For experimental details refer to Methods. Original traces of four rings of rabbit aorta obtained from a single animal are shown. The rings were precontracted with phenylephrine (P, 0.3 μ M). H_2O_2 (0.75 mM) induced the relaxation of the rings denuded of endothelium (E (-)). Addition of H_2O_2 (0.75 mM) to the baths containing intact rings (E (+)) elicited a biphasic response. A small, brief contraction was followed by a pronounced relaxation. Methylene blue (MeB, 10 μ M) reversed the relaxation of both intact and denuded ring ($n = 6$). N^G-monomethyl-L-arginine (L-NMMA, 300 μ M) had no effect on the H_2O_2 -induced relaxation of the denuded ring ($n = 7$). However, it partially reversed H_2O_2 -induced relaxation of endothelium-intact rings ($n = 7$). N^G-nitro-L-arginine methyl ester (L-NAME, 30 μ M) exerted effects similar to those of L-NMMA (300 μ M). L-NAME reversed the relaxations induced by H_2O_2 in intact rings and did not affect the relaxations of rings containing no endothelium ($n = 5$, not shown). Traces are typical of n different experiments.

the relaxant action of H_2O_2 and their inhibitory action was reversed in the presence of 2 mM L-Arg, but not D-Arg. H_2O_2 -induced relaxations of intact rings in the presence of MeB (10 μ M) were reduced as compared to those of rings denuded of endothelium, indicating that MeB inhibited both the endothelium-dependent and endothelium-independent components of the relaxant action of H_2O_2 (Figure 3). H_2O_2 did not affect the tone of intact or denuded rings in the presence of catalase (100 u ml⁻¹, $n = 3$, data not shown). In contrast, superoxide dismutase (50 u ml⁻¹, $n = 6$) did not change the relaxant activity of H_2O_2 (0.3–1 mM) in either intact or denuded rings. Interestingly, in the presence of L-NAME (30 μ M, $n = 11$), L-NMMA (300 μ M, $n = 11$) or MeB (10 μ M, $n = 7$) the initial contraction induced by H_2O_2 was also abolished (data not shown).

The effect of H_2O_2 on the functional integrity of endothelium

In order to establish whether concentrations of H_2O_2 used in this study affect the functional integrity of endothelium of the rabbit aorta, we investigated the effects of pretreatment of the rings of rabbit aorta with H_2O_2 (1 mM) on the endothelium-dependent relaxation induced by ACh. In these experiments, the H_2O_2 -induced relaxation of intact rings precontracted with phenylephrine (0.2–0.3 μ M) was recorded. In control rings the addition of H_2O_2 was omitted. Afterwards, the rings were washed for 1 h with fresh Krebs buffer and, after again contracting the rings with phenylephrine (0.2–0.3 μ M), concentration-response curves to ACh (0.01–3 μ M) were obtained. As shown on Figure 4, pretreatment of the rings with H_2O_2 did not affect subsequent ACh-induced relaxation.

Because we suspected that H_2O_2 -induced peroxidation of lipids might contribute to the effects we had observed we investigated the effect of the active (-)-racemate (U-83836E) of a potent 2-methylaminochroman lipid peroxidation inhibitor U-78517F (Hall *et al.*, 1991) on the vasorelaxant

activity of H_2O_2 . U-83836E (1–50 μM) did not affect the tone of either intact or endothelium-denuded rings of rabbit aorta ($n = 4$, data not shown). The preincubation of rings for 20 min with U-83836E at concentrations (30–50 μM) which effectively inhibit iron-catalysed lipid peroxidation (Hall *et al.*, 1991), did

Figure 3 Prevention of inhibitors of NO synthase and methylene blue of endothelium-dependent component of H_2O_2 -induced relaxation. For experimental details see Methods. The responses of phenylephrine (0.3 μM)-precontracted rings of rabbit aorta induced by H_2O_2 (0.75 mM) are shown. The H_2O_2 -induced relaxations of rings containing undamaged endothelium (E (+), solid bars) were significantly greater than those of rings denuded of endothelium (E (-), open bar) ($n = 15$). Preincubation of the intact rings (20 min) with the inhibitors of NO synthase, N^G -monomethyl-L-arginine (L-NMMA, 300 μM , $n = 11$) or N^G -nitro-L-arginine methyl ester (L-NAME, 30 μM , $n = 11$), blocked the endothelium-dependent component of the relaxant action of H_2O_2 . The inhibitory action of both L-NAME and L-NMMA was reversed in the presence of L-arginine (L-Arg, 2 mM, $n = 5$). D-Arginine (D-Arg, 2 mM) did not affect the inhibitory action of L-NAME or L-NMMA ($n = 6$). Preincubation of intact rings with methylene blue (MeB, 10 μM) inhibited the H_2O_2 -induced relaxation to the level below that observed in the tissues denuded of endothelium ($n = 7$) indicating that MeB blocks both the endothelium-dependent and the endothelium-independent components of the relaxation induced by H_2O_2 . Bars represent means $\pm$ s.e. mean of n different experiments. *Bonferroni $P < 0.01$ as compared to E(-).

Figure 4 The lack of toxic effect of H_2O_2 on the endothelium on the rabbit aorta. For experimental details see Methods. Rings of rabbit aorta were precontracted with phenylephrine (0.2 μM) and H_2O_2 (1 mM) was added to some of the baths. After recording the relaxations induced by H_2O_2 , the rings were washed with fresh Krebs buffer for 1.5 h. Then, following contraction of tissues with phenylephrine (0.2 μM), the relaxant responses induced by increasing concentrations of acetylcholine (ACh, 0.01–3 μM) were recorded. The ACh-induced relaxations of the rings treated with H_2O_2 (1 mM, $\bigcirc$ — — $\bigcirc$) were not different from those of the control rings ($\bullet$ — — $\bullet$). Values represent means $\pm$ s.e. mean of $n = 5$ experiments.

not affect the relaxant responses elicited by H_2O_2 (1 mM) in either intact and denuded rings ($n = 4$, data not shown).

The effect of H_2O_2 on the efflux of cyclic GMP from the rings of rabbit aorta

Incubation of rings of rabbit aorta in Krebs buffer containing IBMX (0.1 mM), an inhibitor of phosphodiesterases, resulted in the accumulation of cyclic GMP in the incubation buffer. As depicted in Figure 5, the concentrations of cyclic GMP attained after 20 min incubation were 5 fold higher if intact endothelium was present. H_2O_2 (1 mM) augmented the efflux of cyclic GMP from denuded rings and, to a significantly greater extent, from rings with intact endothelium. Moreover, the endothelium-dependent component of cyclic GMP efflux was abolished in the presence of L-NAME (30 μM) (Figure 5). As seen in Table 1, the presence of intact endothelium resulted in 4 fold larger net stimulation of cyclic GMP release induced by H_2O_2 . Importantly, ACh (0.3 μM) did not affect the efflux of cyclic GMP from denuded rings, demonstrating the complete removal of endothelium. ACh (0.3 μM) did, however, potentiate the efflux of cyclic GMP from rings with intact endothelium (Table 1).

Figure 5 Enhancement by H_2O_2 of the efflux of cyclic GMP from rings of rabbit aorta. The results of experiments are shown (described in detail in Methods), in which the concentration of cyclic GMP released from the rings of rabbit aorta into the incubation medium was measured by RIA. Columns represent the concentrations of cyclic GMP, expressed as means $\pm$ s.e. means of $n = 12$ –18 experiments, for intact (solid columns) and endothelium-denuded rings (open columns). Hatched columns represent results of experiments performed in the presence of N^G -nitro-L-arginine methyl ester (L-NAME, 30 μM). Incubation of the rings in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.1 mM) for 20 min resulted in detectable levels of extravascular cyclic GMP which were significantly enhanced if intact endothelium was present. H_2O_2 (1 mM) resulted in enhanced efflux of cyclic GMP from denuded rings and, to a significantly greater extent, from endothelium-intact rings. The endothelium-dependent component of the efflux of cyclic GMP was abolished in the presence of L-NAME (30 μM). * $P < 0.05$ when compared to the appropriate control; § $P < 0.05$ as compared to rings without endothelium.

Table 1 Net stimulation of cyclic GMP efflux from the rings of rabbit aorta by H_2O_2 and acetylcholine (ACh)

Presence of endothelium	H_2O_2 (1 mM)	ACh (0.3 μM)
Denuded [E (-), $n = 12$]	$5.1 \pm 1.2^*$	0.6 ± 0.4
Intact [E (+), $n = 18$]	$21.7 \pm 4.2^{*\S}$	$6.7 \pm 1.5^{*\S}$

For experimental details refer to Methods. Values represent net increases in the concentration of extravascular cyclic GMP (fmol mg^{-1}), as compared with the appropriate controls, and are expressed as means $\pm$ s.e. means of n rings. H_2O_2 (1 mM) caused a significant increase in the release of cyclic GMP from both the intact and endothelium-denuded rings, while ACh (0.3 μM) increased cyclic GMP efflux only in the presence of endothelium. * $P < 0.002$ as different from 0 by two-tailed one sample t test, § $P < 0.01$ as compared to E (-) by two-way unpaired t test.

Comparison of the relaxant action of H_2O_2 with the effects of organic peroxides

Stable organic peroxides, *t*-ButHP (0.1–1 mM) and CumHP (0.1–1 mM), caused relaxations of denuded rings similar to those induced by H_2O_2 ($n = 5$). When endothelium was present, the addition of *t*-ButHP or CumHP to the baths induced a biphasic response, consisting of a pronounced contraction followed by relaxation. However, in contrast to the effects of H_2O_2 , the relaxant effects of *t*-ButHP or CumHP were not enhanced in the presence of endothelium ($n = 5$, Figure 6).

Comparison of the effects of H_2O_2 and those of organic peroxides on the efflux of cyclic GMP from the rabbit aorta

As depicted in Figure 7, CumHP (1 mM) did not affect the release of cyclic GMP from the denuded rings and significantly reduced its release from the rings containing intact endothelium. *t*-ButHP (1 mM) slightly enhanced the release of cyclic GMP from denuded rings. Its effect was ca. 50% of that of H_2O_2 . In the presence of endothelium, *t*-ButHP (1 mM) also decreased the efflux of cyclic GMP. These results suggest that the initial contraction of the endothelium-intact rings of rabbit aorta induced by organic peroxides was due to the inhibition of endothelial NO production, most likely through damage caused to the endothelium. Consistent with this notion are observations that ACh-induced relaxations of intact rings treated with CumHP (1 mM) were completely abolished, and those of rings treated with *t*-ButHP (1 mM) were significantly attenuated ($n = 3$, data not shown).

The effect of exogenous donors of NO on H_2O_2 -induced relaxation in the rabbit aorta

Smooth muscle of rings containing intact endothelium is under the continuous influence of basally released NO, which attenuates the contraction induced by phenylephrine. Thus, we tested the hypothesis that the endothelium-dependent component of H_2O_2 -induced relaxation is due to the potentiation by H_2O_2 of the vasorelaxant action of basally released NO within

Figure 6 Comparison of the relaxant effects of H_2O_2 and the stable organic peroxides *tert*-butyl hydroperoxide and cumene hydroperoxide. For experimental details refer to Methods. Original traces of six rings of rabbit aorta obtained from a single animal are shown. The upper traces show the responses of rings denuded of endothelium (E (-)), while those of rings with intact endothelium (E (+)) are shown below. H_2O_2 (1 mM), *tert*-butyl hydroperoxide (*t*-ButHP, 1 mM) and cumene hydroperoxide (CumHP, 1 mM) elicited comparable relaxation of the denuded rings. In the presence of intact endothelium, addition of all tested peroxides resulted in a biphasic response, which consisted of a contraction followed by relaxation. In the presence of H_2O_2 , this initial contraction was small and of short duration while the following relaxation was pronounced and 2–3 fold larger than in the absence of endothelium. In contrast, *t*-ButHP- and CumHP-induced contractions were of much greater extent and duration and were followed by relaxations of a magnitude similar to those induced by organic peroxides in the denuded rings. This figure is representative of $n = 5$ similar experiments.

the smooth muscle. To accomplish this, we investigated the vasorelaxant activity of H_2O_2 (1 mM) in endothelium-denuded rings after the titration of their tone to levels approximating that of rings obtained from the same animals and possessing an intact endothelium. Titrations were performed by the sequential addition of the NO donors SIN-1 (30–60 nM), glyceryl trinitrate (30–80 nM) or sodium nitroprusside (30 nM). However, as shown in Figure 8, the vasorelaxant action of H_2O_2 (1 mM) in the presence of NO donors was not different from that of control rings without endothelium.

Figure 7 Comparison of the effects of H_2O_2 and those of organic peroxides on the efflux of cyclic GMP from the rings of rabbit aorta. The results of a series of experiments are shown (described in detail in Methods), in which the concentration of cyclic GMP released from the rings of rabbit aorta into the incubation medium was measured by RIA. Columns represent net change of cyclic GMP concentration in the incubation buffer in the presence of cumene hydroperoxide (CumHP, 1 mM), *tert*-butyl hydroperoxide (*t*-ButHP, 1 mM) or H_2O_2 (1 mM) and are expressed as means $\pm$ s.e. means of $n = 12$ rings. CumHP (1 mM) did not affect the efflux of cyclic GMP from denuded rings (open columns) while it significantly reduced the efflux from rings containing intact endothelium (solid columns). *t*-ButHP (1 mM) slightly enhanced the release of cyclic GMP from denuded rings. In the presence of endothelium, *t*-ButHP (1 mM) decreased the efflux of cyclic GMP. H_2O_2 (1 mM), on the other hand, significantly enhanced cyclic GMP efflux from both intact and denuded rings. * $P < 0.05$ as different from 0 by two-way, unpaired one sample *t* test.

Figure 8 Lack of effect of NO donors on H_2O_2 -induced relaxation in the rabbit aorta. For experimental details see Methods. The relaxant responses induced by H_2O_2 (1 mM) in endothelium-intact (E (+), solid column) and denuded (E (-), open column) rings of rabbit aorta are shown. In this series of experiments, the tone of denuded rings ($99.7 \pm 12.8\%$ of KCl-induced contraction, $n = 5$) was reduced to the level of rings containing endothelium ($71.9 \pm 4.5\%$ KCl; $n = 10$ from 5 separate animals) by the addition of increasing concentrations of 3-morpholinodimethylamine (SIN-1, 30–60 nM; $68.0 \pm 10.2\%$ KCl; $n = 5$), glyceryl trinitrate (GTN, 30–80 nM; $73.2 \pm 12.6\%$ KCl; $n = 5$) or sodium nitroprusside (SN, 30 nM; $67.8 \pm 10.8\%$ KCl; $n = 5$). H_2O_2 -induced relaxation was enhanced in rings with intact endothelium but not in the denuded rings relaxed with NO-donors. Values represent means $\pm$ s.e. means of n different rings. *Bonferroni $P < 0.01$, as compared to E(-) as well as SIN-1, GTN or SN.

Discussion

Within the cardiovascular system, the major source of H_2O_2 is the activated PMN. A significant, so called marginated, pool of PMNs remains in close contact with endothelial cells. Importantly, in response to tissue mediators of inflammation, PMNs adhere to endothelial cells and crawl along their surface towards interendothelial junctions. The adhesion of PMNs is concomitant with their activation and leads to a massive release of H_2O_2 and other oxygen-derived species into a micro-compartment between plasma membranes of adjacent cells. Therefore, the endothelial cells may be exposed to very high local concentrations of H_2O_2 .

We have investigated the effects of H_2O_2 on the tone of intact and endothelium-denuded rings of rabbit aorta. H_2O_2 , at concentrations of 0.1–1 mM, elicits a concentration-dependent relaxation of both intact and denuded rings. However, the relaxant responses of rings containing undamaged endothelium were 2–3 fold larger than those of denuded rings. This indicates that in the rabbit aorta, the relaxant action of H_2O_2 has endothelium-dependent and endothelium-independent components.

The major novel finding of this paper is that the endothelium-dependent component of the relaxant action of H_2O_2 is mediated through endogenous NO. This conclusion is well supported by several lines of experimental data. The relaxation of endothelium-intact rings induced by H_2O_2 was reversed by L-NAME or L-NMMA, inhibitors of NO synthase (Palmer *et al.*, 1988b; Mülsch & Busse, 1990), while the relaxation of denuded rings was unaffected. Preincubation of endothelium-intact rings with L-NAME or L-NMMA abolished the endothelium-dependent component of H_2O_2 -induced relaxation. The effects of inhibitors of NO synthase were specific because their action could be reversed in the presence of high concentrations of L-Arg, a physiological substrate for NO synthase, while the D-stereoisomer of arginine was inactive. It is important to note that preincubation with L-NAME and L-NMMA did not affect the relaxant responses of denuded rings. Moreover, the relaxations induced by H_2O_2 were also blocked by MeB, an inhibitor of the soluble guanylate cyclase, activation of which is responsible for the vasorelaxant action of NO. However, in contrast to the inhibitors of NO synthase, MeB blocked both the endothelium-dependent and endothelium-components of the relaxant action of H_2O_2 , the implication being that both components depend upon the stimulation of guanylate cyclase within the vessel wall.

Conclusions derived from our bioassay experiments are supported by the measurements of the release of cyclic GMP from rabbit aortic rings. It is well established that a portion of cyclic GMP escapes the action of phosphodiesterases and can be detected extracellularly. The extracellular accumulation of cyclic GMP following stimulation of guanylate cyclase has been observed in isolated segments of rat aorta (Schini *et al.*, 1989), bovine cultured endothelial and rat vascular smooth muscle cells (Hamet *et al.*, 1989; Feelisch & Kelm, 1991), dog kidney epithelial cell line MDCK (Woods & Houslay, 1991), rat cerebellar (Tjörnhannar *et al.*, 1986), and liver slices (Tjörnhannar *et al.*, 1983) as well as rat pineal glands (O'Dea *et al.*, 1978). Moreover, the release of cyclic GMP from aortic rings is closely correlated with intracellular levels of cyclic GMP (Zembowicz *et al.*, unpublished observations). Consistent with these observations are our results showing that the incubation of rings of rabbit aorta in the presence of an inhibitor of phosphodiesterases resulted in an accumulation of cyclic GMP in the incubation buffer which was markedly enhanced in the presence of intact endothelium. H_2O_2 enhanced the levels of cyclic GMP released from the denuded rings which indicates that H_2O_2 enhances the activity of a soluble guanylate cyclase present in the smooth muscle cells of the rabbit aorta. In the presence of intact endothelium, H_2O_2 -induced efflux of cyclic GMP was significantly augmented, an observation which supports

the proposed endothelium-dependent component of guanylate cyclase stimulation induced by H_2O_2 . This endothelium-dependent component of H_2O_2 -induced release of cyclic GMP, like that of the endothelium-dependent component of H_2O_2 -induced relaxation, was abolished after the inhibition of endothelial NO synthase by L-NAME.

It is known that H_2O_2 activates the soluble guanylate cyclase present in the smooth muscle of the bovine aorta by a mechanism that involves its metabolism by catalase (Wolin & Burke, 1987). Consistent with this hypothesis is the inhibition of H_2O_2 -induced relaxation of bovine pulmonary artery by ethanol (Burke-Wolin & Wolin, 1990), which decreases the formation of compound I species of catalase. It is not implausible that the mechanism of endothelium-independent relaxation induced by H_2O_2 in the rabbit aorta also involves the catalase-dependent activation of the soluble guanylate cyclase.

The results of our experiments discussed above clearly establish that the endothelium-dependent component of the vasorelaxant action of H_2O_2 was dependent on the activity of endothelial NO synthase. This effect could be due to the enhancement of the activity of endothelial NO synthase by H_2O_2 or due to a potentiation of vasorelaxant action of basally released NO at the level of the smooth muscle. In order to resolve this, we investigated the relaxant activity of H_2O_2 in denuded rings, the tone of which was decreased by the exogenous donors of NO, SIN-1, glyceryl trinitrate or sodium nitroprusside, to the level induced by NO released from the endothelium of control tissues. Donors of NO did not affect H_2O_2 -induced relaxation. These results strongly support the hypothesis that H_2O_2 enhances endothelial biosynthesis of NO rather than its action in the vascular smooth muscle and implies an augmentation of the activity of NO synthase.

Like H_2O_2 , the stable organic peroxides CumHP and *t*-ButHP induced an endothelium-dependent contraction of the intact rings of rabbit aorta that was followed by a relaxation. However, the initial contractile response induced by organic peroxides was much larger than that induced by H_2O_2 . In contrast to H_2O_2 , the vasorelaxant activity of *t*-ButHP and CumHP was not augmented in the presence of intact endothelium. Thus, the activation of NO synthase induced by H_2O_2 is not a general property of peroxides, but is a phenomenon specific to H_2O_2 . Interestingly, both CumHP and *t*-ButHP reduced the efflux of cyclic GMP from the intact rings. These results may reflect damage to the endothelium induced by these organic peroxides. Finally, CumHP did not affect and *t*-ButHP only slightly enhanced, the efflux of cyclic GMP from denuded rings. This finding suggests that mechanisms other than the activation of soluble guanylate cyclase are involved in the endothelium-independent relaxation induced by organic peroxides.

As already discussed, our results demonstrate that H_2O_2 enhances endothelial synthesis of NO. However, the exact mechanism responsible for the activation of NO synthase by H_2O_2 has not yet been established. It is known that many biological actions of H_2O_2 are mediated by hydroxyl radicals formed during the iron-catalyzed Haber-Weiss reaction of H_2O_2 with O_2^- (Haber & Weiss, 1934; McCord & Day, 1978). However, in contrast to catalase, superoxide dismutase did not affect H_2O_2 -induced endothelial NO synthesis. This implies that the effects of H_2O_2 are not due to the production of hydroxyl radicals as a consequence of its interactions with O_2^- present in oxygenated Krebs buffer.

Exposure of porcine cultured endothelial cells to H_2O_2 at concentrations as low as 100 μ M caused a time-dependent augmentation of cytosolic calcium concentration which was attenuated in the presence of a lipid peroxidation inhibitor U-78517F (Kimura *et al.*, 1992). Thus, we considered lipid peroxidation-induced increase in cytosolic calcium concentration as a possible mechanism of the augmentation of endothelial NO synthase activity by H_2O_2 . However, the active racemate of U-78517F, U-83836E (Hall *et al.*, 1991),

did not affect endothelium-dependent or endothelium-independent components of H₂O₂-induced relaxation. It should be noted that these results do not exclude the possibility that H₂O₂ activates endothelial cells and increases levels of intracellular calcium by a mechanism not involving lipid peroxidation.

Another possibility is suggested by the experiments of Boucher *et al.* (1992) who demonstrated that a number of haemoproteins in the presence of H₂O₂ or other peroxides can catalyze the formation of NO and citrulline from L-HOArg, an intermediate in the biosynthesis of NO from L-arginine (Stuehr *et al.*, 1991; Zembowicz *et al.*, 1991). Consistently, L-HOArg can be metabolized to NO in cultured bovine endothelial cells by a pathway that is not blocked by inhibitors of NO synthase (Zembowicz *et al.*, 1991; 1992). Thus, H₂O₂ may act upon L-HOArg formed by NO synthase and convert it to NO in a haem-dependent manner. Interestingly, NO synthase is also a haemoprotein (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992). Thus, the haem involved in this putative reaction might be the one present in NO synthase.

Whatever mechanisms are involved, H₂O₂-induced augmentation of endothelial NO synthesis may be part of the complex physiological or pathophysiological interactions between activated PMNs and the vessel wall. NO released by H₂O₂ may react with O₂⁻ to form peroxynitrite which then produces cytotoxic hydroxyl radicals (Beckman *et al.*, 1990) thereby contributing to H₂O₂-induced endothelial injury. However, the NO-dependent relaxation induced by H₂O₂ occurred before any irreversible impairment of endothelial cell function, assessed as the ability of endothelial cells to release NO after subsequent stimulation with ACh, could be observed. This suggests that H₂O₂-induced release of NO may also play a regulatory role. NO may oppose the

vasoconstrictor effects of other PMN-derived mediators such as O₂⁻, thromboxane A₂, peptidoleukotrienes (Goldstein *et al.*, 1978; Mullane *et al.*, 1987; Ohlstein & Nichols, 1989) or vasoactive peptides such as angiotensin II or endothelin-1 generated in the circulation of PMN-derived proteases (Wintroub *et al.*, 1984; Dzau *et al.*, 1987; Kaw *et al.*, 1992). Moreover, NO exerts an inhibitory action on many functions of the PMN. SIN-1, an exogenous donor of NO, inhibits the release of lysosomal enzymes and the generation of superoxide anion (O₂⁻) by human PMNs stimulated by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Schröder *et al.*, 1990). Inhibitors of NO synthase have been shown to enhance adhesion of PMNs to the endothelium of the mesenteric venules in the cat *in vivo* (Kubes *et al.*, 1991). Moreover, nitric oxide inhibits the chemotaxis and adhesion to cultured endothelial cells of fMLP-stimulated monocytes (Bath *et al.*, 1991). Thus, the augmentation of the formation of NO by H₂O₂ may serve as a negative feedback mechanism by which the activation of PMNs adhering to the endothelium is modulated by endothelium-derived NO.

We conclude that H₂O₂-induced relaxation in the rabbit aorta has both endothelium-dependent and independent components. The endothelium-dependent component of the relaxant action of H₂O₂ is due to enhanced endothelial synthesis of NO. These findings also suggest that H₂O₂-induced release of NO may play a role in interactions between PMNs and the vessel wall.

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