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Role of nitric oxide and free radicals in the contractile response to non-preactivated leukocytes

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Abstract

Previous studies from our laboratory have shown that nitric oxide (NO) can reduce the release of free radicals from activated leukocytes. The aim of this study was to assess the role of endothelium-derived nitric oxide and leukocyte-derived free radicals in the contractile response to non-preactivated leukocytes. Vessel tension studies were performed in rabbit endothelium-intact aortic vessel rings precontracted with 5-hydroxytryptamine (1 μM). Addition of leukocytes isolated from rabbit blood were added to the rings in increasing concentrations (10^3–10^6 cell ml^{-1}) under control conditions and in the presence of L-nitroarginine methyl ester (L-NAME 1 mM), D-NAME (1 mM), or superoxide dismutase (100 U ml^{-1}). The responses to superoxide radical (generated by xanthine plus xanthine oxidase, X/XO), hydrogen peroxide, hypochlorite and peroxynitrite were also assessed. The nature of the free radicals released from non-activated isolated leukocytes, zymosan-stimulated leukocytes (in whole blood) and isolated vessel rings was assessed using luminol-enhanced chemiluminescence. Cumulative addition of leukocyte suspensions to aortic rings caused a concentration-dependent contractile response which was abolished by preincubation of the vessel ring with L-NAME. D-NAME and superoxide dismutase were without effect. All the free radicals tested produced a relaxation of the precontracted aortic ring. The response to X/XO was not affected by superoxide dismutase, but abolished by catalase. The responses to hydrogen peroxide and hypochlorite were both found to be dependent upon the presence of endothelium and NO. The response to peroxynitrite was endothelium-independent and was blocked by methylene blue. While the main free radical released from unstimulated leukocytes and vessel rings was superoxide, the main radical released from activated leukocytes was found to be hypochlorite. These results suggest that the vascular contraction seen in response to non-preactivated leukocytes is due to inhibition, by NO, of the release of free radicals from the leukocytes when activated by contact with the vascular endothelium, thus allowing co-released vasoconstrictor substances to exert their effect. © 1998 Elsevier Science B.V.

Keywords: Free radical; Leukocyte-induced contraction; Nitric oxide (NO)

1. Introduction

Abnormal accumulation of polymorphonuclear leukocytes occurs in the artery wall in a variety of cardiovascular diseases, including atherosclerosis, restenosis and ischaemia. In these conditions, leukocytes may contribute to the evolution of changes in vascular reactivity, including hyper-reactivity and vasospasm. Several studies have shown that preactivated leukocytes induce a contraction when added to artery ring preparations in vitro. The leukocyte-induced contraction was found to be endothelium-dependent and associated with inhibition of the action of vasodilators that act by releasing nitric oxide (NO) from the endothelium (Murohara et al., 1993; De Kimpe et al., 1993). Moreover, preactivated leukocyte-induced vasoconstriction has been reported to be attenuated by superoxide dismutase (Murohara et al., 1993), suggesting that the contractile activity of leukocytes occurs in part via superoxide formation. Other studies have reported an endothelium-dependent contraction in response to non-preactivated leukocytes (Nishida et al., 1990) and an endothelium-independent contraction produced by supernatants from leukocyte suspensions (Sessa and Mullane, 1990; Mugge et al.,

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1993), suggesting that under appropriate conditions different vasoactive mediators are released by leukocytes.

Arterial contraction induced by non-preactivated leukocytes appears to be dependent upon interaction with the adhesion molecules P-selectin and L-selectin in the artery wall (Murohara et al., 1994). Leukocyte adhesion subsequently leads to activation of a group of enzymes that generate reactive short-lived free radicals (Rosen et al., 1995). The enzymes of the respiratory burst form superoxide, which is then converted to peroxide. Myeloperoxidase utilizes superoxide to form hypochlorite. At the level of the endothelial cell, NO synthase forms NO, which can then react with superoxide to give peroxynitrite. The effects of some of these free radicals on artery tone has been investigated. For example, superoxide, generated from xanthine oxidase, causes contraction of artery rings, possibly by destruction of basally-released NO, since the contraction is dependant on the presence of endothelium (Rhoades et al., 1990; Mian and Martin, 1995). In contrast, relaxation of artery rings is produced by both peroxide (Zembowicz et al., 1993; Iesaki et al., 1994; Mian and Martin, 1995) and peroxynitrite (Liu et al., 1994; Wu et al., 1994; Moro et al., 1995). The role of peroxide and peroxynitrite in leukocyte-induced vascular contraction has not been investigated. Hypochlorite has been reported to cause an increase in perfusion pressure in the perfused rat heart (Leipert et al., 1992), but no studies have been carried out to determine its effects on isolated artery rings.

Nitric oxide both prevents the adhesion of leukocytes to the vascular endothelium (Lopez-Belmonte and Whittle, 1995) and modulates the extent of oxygen-derived free radicals generated from leukocytes on activation (Demiryürek et al., 1997). The aim of this study was to study the possible interactions between endothelium-derived NO and leukocyte-derived oxygen free radicals in the contractile responses to non-preactivated leukocytes in artery rings.

2. Materials and methods

2.1. Materials

Sodium citrate, luminol, dextran (MW approx. 500 000), Histopaque 1077®, Hanks' Buffered Salt Solution (HBSS), 5-hydroxytryptamine (5-HT), indomethacin, acetylcholine, L-nitroarginine methyl ester (L-NAME), D-NAME, superoxide dismutase, catalase, sodium azide, D-mannitol and phorbol myristate acetate (PMA) were all obtained from Sigma (Poole, Dorset).

Sodium citrate, dextran, 5-hydroxytryptamine (5-HT), L-NAME, superoxide dismutase, sodium azide, D-mannitol, catalase, xanthine and xanthine oxidase were all dissolved in distilled water and either used within 6 h or (for L-NAME and 5-HT) refrigerated as stock solutions and diluted as required on a daily basis. Luminol was dissolved in 2 M NH_4OH (2.5%) and made up to volume with phosphate buffered 0.9% NaCl (pH 7.4). Indomethacin was prepared in distilled water and 1 M NaOH added dropwise until dissolution occurred.

2.2. Isolation and separation of leukocytes

Male New Zealand White rabbits (2.5–3.5 kg) were anaesthetised with sodium pentobarbitone (60 mg ml^{-1}) containing heparin (500 IU ml^{-1}). The chest was opened and blood was withdrawn from the pulmonary artery into sterile 20 ml syringes containing 2 ml sodium citrate (3.8%). The blood was immediately transferred to 10 ml centrifuge tubes containing 2 ml dextran (6%) and left to sediment at room temperature for 2 h. The leukocyte-rich upper layer was removed and centrifuged at $180 \times g$ for 5 min. The resultant cell pellet was lysed hypotonically with distilled water, to remove contaminating erythrocytes, and layered onto 1 ml Histopaque® (1.077 g ml^{-1} density). A final centrifugation at $200 \times g$ for 20 min yielded a white cell pellet which was resuspended in Hank's balanced salt solution (HBSS). After isolation, cells were counted using a cell counter (Medonic Cellanalyzer CA 460, Sweden) and leukocyte yield adjusted to 5×10^6 cells ml^{-1} with HBSS. Leukocytes were stored at room temperature prior to use.

2.3. Chemiluminescence studies

2.3.1. Measurement of spontaneous chemiluminescence from unstimulated leukocytes and isolated blood vessel rings

Generation of free radicals was measured by luminol-enhanced chemiluminescence using a chemiluminometer (Lumi-vette, Chronolog, Haverton, PA). To characterise the spontaneous release of free radicals from isolated blood vessels, rings of rabbit aorta (2–3 mm in length) were suspended on a wire hook in a cuvette containing 900 ml HBSS. The cuvette was warmed at 37°C for 5 min and luminol (225 μM, final concentration) was added prior to measurement of chemiluminescence for 15 min. To determine the influence of unstimulated leukocytes on free radical generation by vessel rings, the cuvette contained 450 μl HBSS and 450 μl leukocyte suspension (10^6 cells ml^{-1} in HBSS), which were warmed to 37°C separately and combined in the measuring chamber. The influence of endothelium-derived nitric oxide on vascular production of chemiluminescence was assessed by preincubating the vessel ring with L-NAME (1 mM) for 30 min prior to measurement of chemiluminescence. The effects of sodium azide (1 mM) and superoxide dismutase (100 U ml^{-1}) were assessed by adding 1 min prior to chemiluminescence

measurement. Spontaneous chemiluminescence generation by non-stimulated leukocytes was also measured in the absence and presence of superoxide dismutase.

2.3.2. Characterisation of free radicals generated by leukocytes in whole blood

Generation of free radicals from zymosan-stimulated leukocytes was measured in whole blood by mixing 90 μl of whole blood with 810 μl of HBSS in a 1-ml cuvette. Following incubation at 37°C for 5 min, the cuvette was transferred to the measuring chamber and 10 μl zymosan ($\sim 10^7$ particles ml^{-1}, final concentration) and 100 μl luminol (225 μM, final concentration) were added. The total chemiluminescence generated over 15 min was measured. To characterise the free radicals generated by these cells, the following scavengers were added to the cuvette, 1 min prior to the addition of luminol, to achieve the following final concentrations: Mannitol (100 μM; to scavenge hydroxyl radical), sodium azide (10 μM to 1 mM; to inhibit myeloperoxidase), superoxide dismutase (100 U ml^{-1}, to dismutate superoxide), catalase ($3–6 \times 10^3$ U ml^{-1}; to destroy hydrogen peroxide) or superoxide dismutase (100 U ml^{-1}) plus catalase (6×10^3 U ml^{-1}). The effects of the scavengers were quantified by calculating the % reduction in chemiluminescence generated compared to zymosan control. To compensate for any loss in responsiveness of the blood to zymosan with time, control responses to zymosan were repeated every fourth measurement and this value was then employed as the control for the three subsequent measurements.

2.4. Vessel tension studies

Rabbit aortic rings (2–3 mm length) were suspended between two parallel intraluminal wires, one fixed and the other attached to an isometric transducer (FT03 Grass Instrument Division). The rings were placed in 5 ml water baths maintained at 37°C and filled with Krebs solution of the following composition (mM): NaCl, 118.3; KCl 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and glucose, 11.1. The rings were placed under a previously determined resting force of 1 g (Hadoke et al., 1993) and allowed to equilibrate for 1 h. All rings were sensitised by three separate additions of 40 mM KCl. Denudation of the endothelium was achieved by gently rubbing the luminal surface with a roughened wire. Absence of endothelium was confirmed by lack of relaxation in response to 10 μM acetylcholine. All vessel tension experiments were performed in the presence of indomethacin (1 mM) to abrogate any effects of prostanoids. A concentration of 1 μM 5-HT was chosen for experiments involving precontraction of the vessel rings. This concentration produced highly reproducible contractions in intact rings (2.47 ± 0.15 g; $n = 51$), which were not significantly different from rings subjected to either endothelial denudation (2.57 ± 0.28 g; $n = 10$) or pretreatment with either L-NAME (2.41 ± 0.22 g; $n = 34$) or D-NAME (2.89 ± 0.3 g; $n = 11$).

2.4.1. Assessment of vascular responses to unstimulated leukocytes

Artery rings were precontracted with 5-HT (1 μM) and leukocytes were added cumulatively to give concentrations of $10^3–10^6$ cells ml^{-1} in the bath. Where leukocytes or rings were pretreated with L-NAME (1 mM), this was added to either the cell suspension or to the vessel ring 30 min before the construction of the concentration response curve.

2.4.2. Assessment of vascular responses to superoxide radical

Vessel rings were precontracted with 5-HT (1 μM). Superoxide was generated by addition of xanthine plus xanthine oxidase to the organ bath. The influence of increasing concentrations of xanthine (1 μM–1 mM) and xanthine oxidase (0.025–25 mU ml^{-1}) on vessel tone were determined. The effects of superoxide dismutase (100 U ml^{-1}), catalase (1000 U ml^{-1}) and L-NAME (1 mM) on the responses to xanthine (100 μM) plus xanthine oxidase (2.5 mU ml^{-1}) were also assessed.

2.4.3. Assessment of vascular responses to hydrogen peroxide

The responses to hydrogen peroxide (H_2O_2) were assessed in rings precontracted with 5-HT (1 μM) by cumulative addition of H_2O_2 (30 μM–3 mM) to the organ bath. The effects of endothelial denudation and L-NAME (1 mM) on the responses to 100 μM H_2O_2 were also assessed.

2.4.4. Assessment of the vascular responses to sodium hypochlorite

Sodium hypochlorite was employed as a source of hypochlorite radical. Cumulative concentration–response curves to sodium hypochlorite (10 μM–1 mM) were performed on aortic rings precontracted with 5-HT (1 μM) in both intact and endothelium-denuded preparations. Similar responses were assessed in rings under baseline tension to determine any contractile responses to sodium hypochlorite.

2.4.5. Assessment of vascular responses to peroxynitrite

Peroxynitrite was prepared by the method according to Moro et al. (1995). Briefly, an ice-cold solution (50 ml) of $NaNO_2$ (1.8 M) and H_2O_2 (1.8 M) was stirred rapidly. A total of 25 ml of HCl (2.8 M) was then thrown into the solution, followed 1 s later by 25 ml NaOH (4.2 M). This yields peroxynitrite in a concentration range of 200–350 mM (Moro et al., 1995). For the purposes of the present experiments, a concentration of 300 mM was assumed and subsequent dilutions performed on this basis. Increasing

Table 1
The effects of scavengers on spontaneous chemiluminescence generated by non-stimulated leukocytes and isolated vessel rings

Preparation	Chemiluminescence generated (arbitrary units)				
	Control	Superoxide dismutase (100 U ml^{-1})	L-NAME (1 mM)	Na azide (1 mM)	Endothelial denudation
Vessel ring ($n = 8$)	376 ± 41	0[a]	378 ± 68	314 ± 50	85 ± 24[a]
Leukocytes ($n = 9$)	300 ± 76	0[a]			
Vessel plus leukocytes ($n = 9$)	268 ± 56		506 ± 141		88 ± 24[a]
Metal hook ($n = 8$)	129 ± 26				

Values are shown as mean ± S.E.M. of n observations. [a] $P < 0.05$ compared to control (Student's paired t-test).

concentrations of peroxynitrite (10 nM–1 mM) were then added to endothelium-intact aortic rings, either at basal resting tension (to assess contractile responses) or precontracted with 5-HT (1 μM; to assess vasodilator responses). The influence of methylene blue (10 μM) on the vascular responses to peroxynitrite were also assessed.

2.5. Statistics

All results are shown as mean ± S.E.M. The n number refers to the number of aortic rings employed from different rabbits. In all experiments, the contraction produced by 5-HT was taken as 100% and subsequent relaxations expressed as % of the maximal 5-HT induced contraction. Parallel time control rings, which were contracted with 5-HT only, were employed to assess any spontaneous relaxation of the 5-HT contraction.

Statistical significance was assessed by paired t-test, one-way analysis of variance or by two-way analysis of variance (specified in the figure and table legends).

3. Results

3.1. Chemiluminescence studies

Aortic rings suspended in buffer produced a small but measurable chemiluminescence signal which was completely abolished by the addition of superoxide dismutase (Table 1). Neither sodium azide nor L-NAME modified this spontaneous chemiluminescence, while endothelial denudation reduced the chemiluminescence signal to a level similar to that seen with the metal hook alone. Freshly extracted whole blood did not produce a chemiluminescence signal ($n = 8$; data not shown), whereas a suspension of isolated leukocytes produced a chemiluminescence signal similar to that generated by the vessel alone. The chemiluminescence generated by the leukocytes was also totally abolished by the addition of superoxide dismutase (Table 1). When leukocytes were added to the vessel ring, the chemiluminescence generated was similar to that seen with either one alone and was significantly reduced by endothelial denudation. Preincubation of the vessel ring with L-NAME increased the chemiluminescence signal almost twofold, although this just failed to reach statistical significance.

Leukocyte stimulation by zymosan in whole blood generated a large chemiluminescence signal. This signal was almost completely abolished by the inclusion of the myeloperoxidase inhibitor, sodium azide (Fig. 1). Superoxide dismutase and catalase also significantly reduced chemiluminescence, although co-addition of these two scavengers did not result in an enhanced reduction of

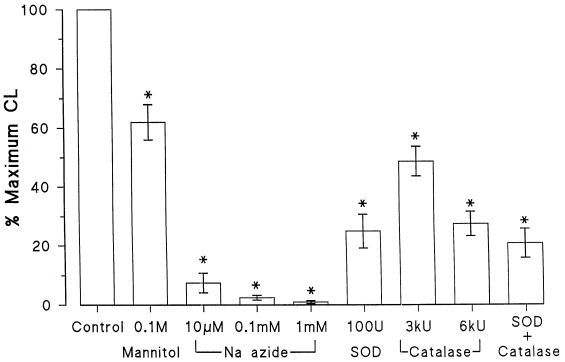

Fig. 1. The effects of the hydroxyl radical scavenger mannitol, the myeloperoxidase inhibitor sodium azide, superoxide dismutase and the H_2O_2 scavenger catalase on chemiluminescence generated by zymosan stimulated leukocytes in rabbit whole blood. Values are mean ± S.E.M. $n = 6$ for each group. $* P < 0.05$ vs. control (one-way analysis of variance).

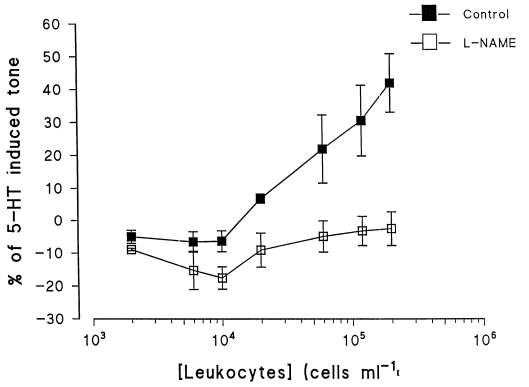

Fig. 2. Concentration response curve of the responses of vessel rings precontracted with 5-HT (1 μM) to unstimulated isolated leukocytes in the absence and presence of L-NAME (1 mM). Values are mean ± S.E.M. Each curve represents data from six vessel rings and leukocytes obtained from different rabbits. The two curves are significantly different ($P < 0.01$ by two-way analysis of variance).

Table 2
The relaxation of aortic rings precontracted with 5-HT (1 μM) by xanthine/xanthine oxidase

[Xanthine] (M)	[Xanthine oxidase] (mU ml^{-1})	% Relaxation of 5-HT induced tone
10^{-6}	0.025	12.3 ± 3.1
10^{-5}	0.25	13.8 ± 4.6
10^{-4}	2.5	30.0 ± 6.5
10^{-3}	25	37.8 ± 12.3

Values are mean ± S.E.M. $n = 6$ for each concentration.

chemiluminescence. Mannitol, the hydroxyl radical scavenger, also significantly reduced chemiluminescence, but to a much lesser extent than the other scavengers studied.

3.2. Vascular responses to unstimulated leukocytes

Cumulative addition of unstimulated leukocytes (10^3–10^6 cells ml^{-1}) caused a concentration-dependent contractile response in rings under baseline force or when precontracted with 5-HT. This contractile effect of the leukocytes was abolished by preincubating the vessel ring with 1 mM L-NAME (Fig. 2). However, preincubation of the leukocytes with L-NAME did not significantly alter the contractile response to the cells. D-NAME (1 mM) had no significant effect on leukocyte-induced contractions when added to the ring; the response to 10^6 cells was 42.1 ± 9.1% of 5-HT induced tone in untreated rings ($n = 7$) vs. 50.7 ± 3.8% in D-NAME treated rings ($n = 4$). Addition of superoxide dismutase (100 U ml^{-1}) to the organ bath immediately before the addition of 6×10^5 leukocytes to the vessel also had no effect on the contractile response of the aortic rings; the leukocytes-induced an increase in 5-HT induced tone of 61.4 ± 10.2% in the absence of superoxide dismutase, compared with 72.3 ± 12.9% in the presence of superoxide dismutase ($n = 8$).

3.3. Vascular responses to free radicals

The response to addition of X/XO to the organ bath was a relaxation of the 5-HT induced tone, the maximum response being approximately 35–40% relaxation (Table 2). The relaxation in response to 100 μM xanthine plus 2.5 U ml^{-1} xanthine oxidase was abolished by pretreatment of the vessel with L-NAME and significantly reduced by catalase (Fig. 3). Endothelial denudation also abolished the response (data not shown). Superoxide dismutase, however, had no effect on the response to X/XO.

Hydrogen peroxide caused a concentration-dependent relaxation of 5-HT induced tone which was significantly attenuated by endothelial denudation (Fig. 4; $P < 0.05$). The relaxant response to 10^{-4} M H_2O_2 (30.5 ± 1.8%) was also significantly reduced by pretreatment of the vessel ring with 1 mM L-NAME (10.0 ± 1.4% relaxation; $P < 0.01$).

Sodium hypochlorite produced a concentration-dependent relaxation of 5-HT induced tone in vessel rings (Fig. 5), but had no effect on vessel tone when applied to rings under resting baseline tension ($n = 3$; data not shown). Endothelial denudation had no effect on the vasorelaxant

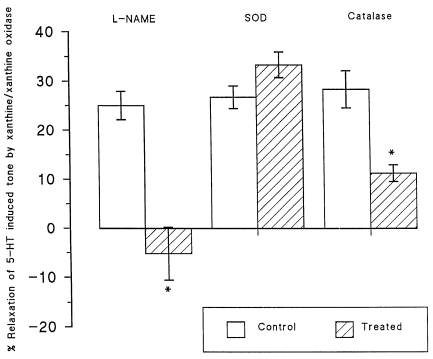

Fig. 3. The effects of L-NAME (1 mM; $n = 8$), superoxide dismutase (100 U ml^{-1}; $n = 8$) and catalase (1000 U ml^{-1}; $n = 7$) on changes in vessel tone induced by xanthine (100 μM) plus xanthine oxidase (2.5 mU ml^{-1}) in rabbit aortic rings precontracted with 5-HT (1 μM). Values are mean ± S.E.M. n numbers relate to vessel rings from different rabbits. * $P < 0.05$ compared with the corresponding control value (paired t-test).

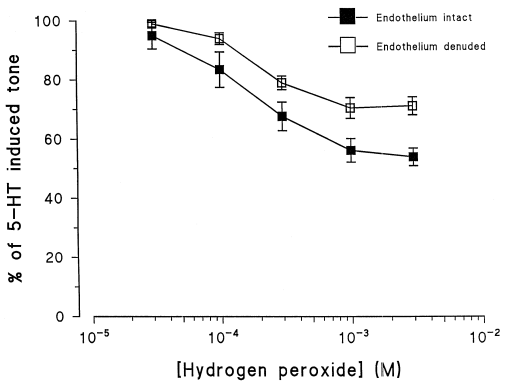

Fig. 4. Concentration–response curve of the relaxant responses of endothelium-intact and endothelium-denuded rabbit aortic rings precontracted with 5-HT (1 μM) to H_2O_2. Values are mean ± S.E.M. Each curve represents data from six vessel rings obtained from different rabbits. The two curves are significantly different ($P < 0.01$, two-way analysis of variance).

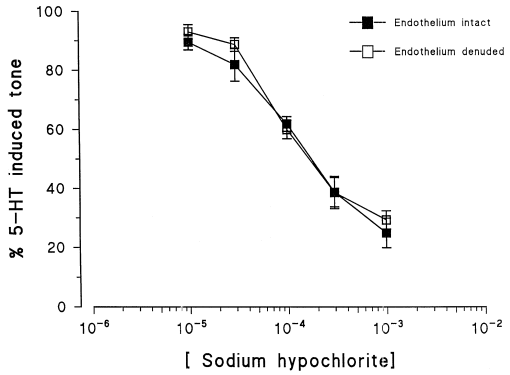

Fig. 5. Concentration–response curve of the relaxant responses of endothelium-intact and endothelium-denuded rabbit aortic rings precontracted with 5-HT (1 μM) to sodium hypochlorite. Values are mean ± S.E.M. Each curve represents data from six vessel rings obtained from different rabbits. The two curves are not significantly different (two-way analysis of variance).

effect of sodium hypochlorite (Fig. 5). When the concentration response curve to sodium hypochlorite was repeated in vessel rings precontracted with KCl (30 mM) there was no effect on vascular tone ($n = 3$, data not shown).

Addition of increasing concentrations of peroxynitrite (up to 1 mM) to vessel rings under baseline tension had no effect on tone ($n = 6$; data not shown). However, when peroxynitrite was added to vessel rings precontracted with 5-HT a biphasic relaxation was observed. The initial response was a transient relaxation which persisted for 2 min, followed by a sustained relaxation with a slower onset. Since previous studies with peroxynitrite solution have shown that the initial response is due to the action of peroxynitrite, while the sustained response is due to decomposed peroxynitrite (primarily nitrite; Wu et al., 1994), only the data for the rapid response is shown (Fig. 6). In the presence of methylene blue (which potentiated the 5-HT induced contraction by approximately 60%), peroxynitrite no longer produced any relaxation of the precontracted vessel (Fig. 6). In vessel rings under resting tension, methylene blue caused a contraction which was not influenced by subsequent addition of peroxynitrite ($n = 6$; data not shown).

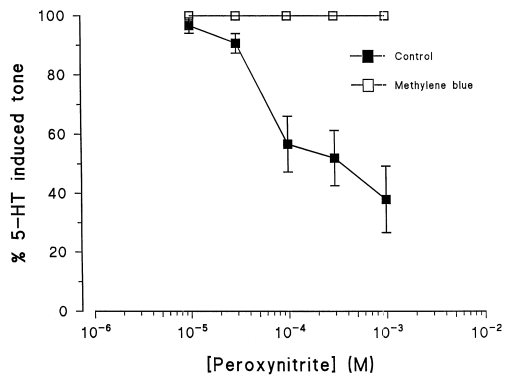

Fig. 6. Concentration–response curve of the relaxant responses of endothelium-intact rabbit aortic rings precontracted with 5-HT (1 μM) to peroxynitrite in the absence and presence of methylene blue (10 μM). Values are mean ± S.E.M. Each curve represents data from six vessel rings obtained from different rabbits. The two curves are significantly different ($P < 0.01$, two-way analysis of variance).

4. Discussion

A number of studies have shown previously that isolated leukocytes cause contractile responses in isolated vessels from different species and different vascular beds. Studies with preactivated leukocytes have demonstrated that the contractions observed can be attenuated by superoxide dismutase and follow a similar time-course to generation of free radicals by the leukocytes following stimulation, resulting in the conclusion that superoxide plays a major role in the contractile responses (Ohlstein and Nichols, 1989; Murohara et al., 1993). These contractions are thought to be due to inactivation of basal NO production by superoxide, since both inhibition of endothelial NO production and endothelial denudation can attenuate the response (Murohara et al., 1993). Despite the preactivation of the cells, the contractile response still appears to require leukocyte–endothelial cell interaction since monoclonal antibodies to macrophage-1 antigen (Mac-1; on the leukocyte) and intercellular adhesion molecule-1 (ICAM-1; on the vessel), and adenosine (via an action at adenosine A_{2A} receptors) prevent both leukocyte adhesion to vascular endothelium and the contractile response to stimulated leukocytes (Minamino et al., 1996a,b).

The mechanisms underlying the contractile responses to non-preactivated leukocytes has not been investigated as thoroughly. Early studies which assessed the effects of supernatants from unstimulated leukocytes demonstrated that this also produced a contractile response in vessel rings. Sessa and Mullane (1990) found that the contraction was endothelium-independent, not due to a free radical and was also observed following prior stimulation. They also found a stable peptide-like substance in the supernatant which could be responsible for the contraction. Sobey et al. (1992), however, found that if the leukocytes were primed with TNF-α the supernatant produced a contraction which was endothelium-dependent. Thus, the level of activation, if any, of the leukocytes appears to influence the nature of the contractile factors released from leukocytes. Few studies so far, however, have investigated the vascular responses to non-preactivated leukocytes, all of which have shown that a contraction develops in response to adding increasing concentrations of leukocytes to isolated vessel rings. One of these studies (De Kimpe et al., 1992) demonstrated the ability of non-preactivated bovine leukocytes to induce a contraction alone and to enhance

contractile responses to noradrenaline which could be prevented by superoxide dismutase and by endothelial denudation. Nishida et al., 1990 also demonstrated that leukocyte-induced contractions were endothelium-dependent, and involved release of vasoconstrictor leukotrienes from the leukocytes following a 'metabolic' interaction between the leukocyte and the endothelial cell. Furthermore, Murohara et al., 1994 have shown that a leukocyte–endothelial cell interaction involving the selectins was essential for the contractile response, since it was inhibited by monoclonal antibodies to P- and L-selectin.

The results of this study have taken these findings one step further by demonstrating the importance of endothelium-derived nitric oxide in the contractile response to non-preactivated leukocytes. The nitric oxide synthase inhibitor L-NAME (but not the inactive isomer, D-NAME) abolished the contractile response observed following cumulative administration of non-preactivated leukocytes to rabbit aorta, suggesting that the contractile response is dependent upon nitric oxide. In contrast to published findings with preactivated leukocytes, superoxide dismutase had no effect on the contractile response. This suggests that the mechanism of the contraction in response to non-preactivated cells is different to that seen with preactivated leukocytes in that superoxide is not involved. One further possibility, which cannot be ruled out entirely, however, is that the contractile responses may be due to platelet contamination of the leukocyte suspension, since it has been shown that under some circumstances platelets can cause contractions (Sessa and Mullane, 1990). However, the isolation procedure employed in this study results in very low platelet concentrations ($\sim$ 1.4–2.2 platelets per leukocyte) which is probably too low to generate contractions of the amplitude seen in our experiments.

The results from the chemiluminescence study show that, under basal conditions, both leukocytes and isolated vessel rings generate low levels of free radicals which are completely absent in the presence of superoxide dismutase. This is indicative of basal superoxide production. However, when the leukocytes (in whole blood) are activated with zymosan, the nature of the free radicals alters in that the predominant radical generated is HOCl, since the chemiluminescence signal was completely abolished by the myeloperoxidase inhibitor, sodium azide, which has been reported to have no effect on superoxide generation (Lucas and Solano, 1992). This finding is similar to previous findings from our laboratories in porcine isolated leukocytes stimulated with PMA (Demiryürek et al., 1994). Thus, under conditions where non-preactivated cells are added to a vessel ring, contact with the ring may result in leukocyte activation and the consequent release of free radicals, other than superoxide, which may be responsible for the vasoconstrictor effect.

We, therefore, studied the effects of superoxide, hydrogen peroxide, hypochlorite and peroxynitrite on vessel rings precontracted with 5-HT. In the case of X/XO-generated superoxide, we found a vasorelaxation which was resistant to superoxide dismutase but completely abolished by catalase. This is inconsistent with data in both pulmonary artery (Rhoades et al., 1990) and rat aortic (Mian and Martin, 1995) rings where the responses to either X/XO or hypoxanthine/XO were of a contractile nature and were blocked either partially or completely by superoxide dismutase. The contractile response to superoxide has been attributed to breakdown of endothelial nitric oxide, although studies in the pulmonary artery suggest that it may involve activation of protein kinase C (Jin et al., 1991). In the study by Mian and Martin (1995), the experiments were performed in the presence of catalase to eliminate the effects of peroxide, whereas in the present studies catalase was not included in the X/XO solution. Thus it is most likely that, while X/XO does result in superoxide generation, this rapidly decomposes to peroxide in the absence of catalase and it was, therefore, most probably a response to peroxide that we were observing under our experimental conditions.

Our results with hydrogen peroxide, however, are consistent with the literature. In our experiments, hydrogen peroxide relaxed artery rings precontracted with 5-HT, an effect which was attenuated, but not abolished, by both endothelial removal and L-NAME. Similar results were found by Zembowicz et al. (1993) who found that the relaxant response to hydrogen peroxide has two components, one of which is endothelium-dependent and due to NO release, the other being endothelium-independent and due to guanylate cyclase activation. Other studies, however, have suggested that the endothelium-independent action of hydrogen peroxide is due to interference with the cellular signal mediating the responses to contractile agonists, possibly by suppressing agonist-induced increases in sensitivity to intracellular Ca^{2+}, since it was unable to relax vessel rings precontracted with KCl (Iesaki et al., 1994, 1996). The responses we observed to peroxynitrite are also consistent with the literature, in that it produced a rapid vasorelaxation of precontracted vessel rings which was completely abolished by methylene blue. This response to peroxynitrite has been shown to be endothelium-independent, inhibited by the NO scavenger haemoglobin and potentiated by superoxide dismutase and probably therefore acts via nitrosylation of tissue glutathione by peroxynitrite to generate NO (Liu et al., 1994; Wu et al., 1994; Moro et al., 1995).

Hypochlorite also produced a vasorelaxant effect on 5-HT precontracted vessel rings which was endothelium-independent. However, in contrast to the other radicals tested, there is nothing in the literature of its effects on isolated vessel rings, although it has been shown to increase coronary perfusion pressure in rat isolated perfused hearts (Leipert et al., 1992). What we found was that hypochlorite has no relaxant effect on vessel rings precontracted with KCl, suggesting that it interferes in some way

with agonist-induced contraction, perhaps in a similar way to the effects reported for hydrogen peroxide.

Our findings with the responses of isolated vessel rings to the radicals which we would expect to be released from leukocytes upon activation by contact with vascular endothelium, therefore, do not implicate any of these radicals as being responsible for the contractile responses to non-preactivated leukocytes. However, the fact that the free radicals themselves are vasorelaxant, may explain the mechanism of the leukocyte-induced contraction. Recent studies from our laboratory have shown that nitric oxide donors can reduce free radical generation from porcine activated leukocytes (Demiryürek et al., 1997). Furthermore, in the present experiments, when the vessel ring was incubated with L-NAME the basal release of free radicals was elevated (although this just failed to reach statistical significance) suggesting that endothelial nitric oxide reduces free radical generation by unstimulated leukocytes. NO also modulates leukocyte adhesion, since L-NAME has been shown to enhance adhesion to endothelial cells (Lopez-Belmonte and Whittle, 1995; Niu et al., 1996). Thus, the explanation for the NO-dependent contraction may be that, while non-preactivated leukocytes which come in contact with vascular endothelium are activated to release their cell contents (i.e., free radicals and a range of vasoconstrictor substance such as leukotrienes), in the presence of an intact endothelium the NO produced would reduce the amount of free radicals released from the leukocytes. The vasoconstrictor substances, however, would continue to be released, with the net result of a vasoconstrictor response. Conversely, in the absence of endothelial-derived NO (i.e., in the presence of L-NAME) free radical release (and leukocyte adhesion) would be increased, with the net result that the vasorelaxant effects of the radicals offsets the vasoconstrictor effect of the other substances released from the leukocytes.

In summary, we have shown that the vasoconstrictor effect of non-preactivated leukocytes is dependent upon production of NO from the vascular endothelium. Since the free radicals which are generated from leukocytes upon activation all appear to have vasorelaxant effects, they cannot be responsible for the vascular constriction seen in response to these cells. However, since endothelium-derived NO both reduces free radical generation and adhesion of leukocytes to vascular endothelium, removal of this influence would result in the over-production of vasorelaxant free radical species which would counteract the vasoconstrictor effects of other vasoactive agents released from these cells.

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