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The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide, or bovine retractor penis inhibitory factor

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1 The mechanisms by which haemoglobin and methaemoglobin inhibit the vasodilator actions of glyceryl trinitrate, sodium azide, nitric oxide, and the bovine retractor penis inhibitory factor (IF) were studied on rabbit endothelium-denuded aortic rings.

2 Methaemoglobin was less effective than haemoglobin against each vasodilator, it was more effective at inhibiting the relaxation to azide than that to glyceryl trinitrate.

3 Glyceryl trinitrate was neither bound nor inactivated when passed through columns of haemoglobin-agarose or methaemoglobin-agarose. Azide was reversibly bound but less by haemoglobin-agarose than by methaemoglobin-agarose. Inhibition of the vasodilator actions of glyceryl trinitrate is not attributable therefore to a direct interaction with the haemoproteins, although a small part of the inhibition of azide-induced relaxation by methaemoglobin is likely to be due to a direct interaction.

4 Columns of haemoglobin-agarose were more effective than columns of methaemoglobin-agarose in removing nitric oxide from solution. The greater ability of haemoglobin, compared to methaemoglobin, to inhibit vasodilatation induced by nitrovasodilators may therefore reflect the greater ability of haemoglobin to bind nitric oxide which is the active principle of the nitrovasodilators.

5 Neither the acid-activated nor the inactive forms of IF were bound or inactivated when passed through columns of methaemoglobin-agarose. Neither form of IF was retained on passage through colomns of haemoglobin-agarose, but the resulting activity in the eluates was less than control, was unstable and, unlike the original activity, decayed rapidly on ice. The greater ability of haemoglobin, compared to methaemoglobin, to inhibit vasodilatation induced by IF might therefore reflect the greater ability of haemoglobin to interact with this vasodilator and inactivate it.

Introduction

Nitrovasodilators such as glyceryl trinitrate and azide, endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and bovine retractor penis inhibitory factor (IF) (Gillespie & Martin, 1980) each induce smooth muscle relaxation in association with an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content (Schultz *et al.*, 1977; Rapoport & Murad, 1983; Bowman & Drummond, 1984). The action of the nitrovasodilators is thought to be mediated through the intracellular formation of nitric oxide (Arnold *et al.*, 1977; Katsuki *et al.*, 1977); for azide this is catalysed by the ferric haem-containing enzyme, catalase (Mittal *et al.*, 1975), though for other nitrovasodilators the mechanism by which nitric oxide is formed is less clear. Craven & De Rubertis (1978) have shown that nitric oxide must interact with a ferrous haem moiety linked to soluble guanylate cyclase to stimulate the enzyme. This ferrous haem moiety may be regarded as the receptor for nitric oxide and possibly for endogenous stimulants of soluble guanylate cyclase such as EDRF (Busse *et al.*, 1985). Competition between exogenously added haemoglobin and the ferrous haem moiety on soluble guanylate cyclase for binding of the active principles might explain the ability of haemoglobin to inhibit the smooth muscle relaxation and concomitant increases in cyclic GMP induced by EDRF, the nitrovasodilators and IF (Bowman & Drummond,

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1984; Martin *et al.*, 1985a). This property of haemoglobin is shared with myoglobin but not with reduced cytochrome C (Martin *et al.*, 1985b). Furthermore, methaemoglobin is much less effective than haemoglobin at blocking the actions of EDRF, the nitrovasodilators or IF (Bowman *et al.*, 1982, Martin *et al.*, 1985b). Thus, only ferrous haem proteins with ligand binding properties are inhibitory, strengthening the concept that haemoglobin exerts its inhibitory effects by binding the active principles. This view is supported by the work of Cocks & Angus (1985) who showed that EDRF activity was lost on passage through columns of haemoglobin bound to agarose.

In this study we have explored further the mechanism by which haemoglobin exerts its inhibitory action. We compared the abilities of haemoglobin to those of methaemoglobin, immobilized on agarose beads and packed in columns, to bind or inactivate (1) the nitrovasodilators, glyceryl trinitrate and azide (2) their active principle nitric oxide and (3) IF.

A preliminary account of some of these findings has been published (Martin & Smith, 1985).

Methods

Organ bath studies

The preparation of aortic rings was similar to that described by Furchgott & Zawadzki (1980). Briefly, male New Zealand white rabbits weighing 2 to 3 kg were killed by stunning and exsanguination. The aorta was removed, cleaned of adhering fat and connective tissue, and cut into 2.5 mm wide transverse rings using a razor blade slicing device. Endothelial cells were removed by gently rubbing the intimal surface with a wooden stick for 30 to 60 s. Successful removal of endothelial cells was confirmed later by the inability of acetylcholine (1 μM) to induce relaxation. Rings were then mounted under 2 g resting tension on stainless steel hooks in 5 ml, or in some experiments 20 ml organ baths, and bathed at 37°C in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and disodium EDTA 0.03, and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically using Ormed UFI force transducers, and displayed on an Ormed Multitrace 4 chart recorder. Tissues were allowed to equilibrate for 90 min before experiments were begun, during which time the resting tension was maintained at 2 g. For relaxation studies, submaximal tone (usually 40–70% of maximum) was induced with phenylephrine. Relaxation responses were obtained to cumulative additions of the nitrovasodilators and to single additions of solutions of nitric oxide and of the bovine retractor penis inhibitory factor.

Preparation of bovine retractor penis inhibitory factor

Extracts containing IF were prepared as previously described (Gillespie *et al.*, 1981). Briefly, fresh bovine retractor penis muscles were obtained from the abattoir, finely chopped and extracted overnight in methanol (5 ml g^{-1} tissue) with continuous stirring at 4°C. The extract was then filtered and 60 ml aliquots applied to 3.5 × 0.5 cm columns of Bio-Rad AGl–X8 (formate form). The columns were then washed with 2 × 5 ml of double-distilled water, and eluted with 6 ml of 300 mM sodium chloride solution. The eluates were adjusted to pH 9–9.5, passed through 3.5 × 0.5 cm columns of alumina to remove adenine nucleotides (Bowman *et al.*, 1979) and the resulting eluates were neutralized and stored frozen in aliquots at −20°C for up to three weeks. When prepared in this way, the extracts had a final concentration equivalent to 2 g of tissue per ml. When required, the extracts were acid-activated (Gillespie & Martin, 1980) by adjusting the pH to 2 for 10 min using 5M HCl and subsequently neutralising with lM NaOH. The extracts were kept on ice at all times.

Preparation of solutions of nitric oxide

Nitric oxide gas was prepared in a fume cupboard as described by Miki *et al.* (1977) by reacting equal amounts (300 mg) of sodium nitrite and ferrous sulphate with lM sulphuric acid under an atmosphere of oxygen-free nitrogen. Solutions of nitric oxide were prepared by bubbling 10 ml of nitric oxide gas through 100 ml of degassed HEPES buffer, 5 mM, pH 7.4, containing 10 μM ascorbate. These solutions were used within 1–2 min since nitric oxide rapidly decays in the presence of oxygen to form nitrite. Nitric oxide produces a characteristically transient vasodilator response (Gruetter *et al.*, 1980) and does not oxidize haemoglobin (Antonini & Brunori, 1971), whereas nitrite induces a slowly developing and maintained vasodilatation and oxidizes haemoglobin (Antonini & Brunori, 1971). Results from experiments in which solutions of nitric oxide induced maintained-vasodilator responses or produced oxidation of haemoglobin-agarose columns (detected by a change in colour from red to brown) were discarded.

Preparation of haemoglobin and methaemoglobin

As supplied, bovine haemoglobin Type 1 (Sigma) contains a mixture of ferrous haemoglobin and its oxidized derivative, methaemoglobin. Solutions of haemoglobin or methaemoglobin were prepared by adding to 1 mM solutions of Sigma haemoglobin in distilled water a 10 fold molar excess of sodium dithionite ($Na_2S_2O_4$) or a 2 fold molar excess of potassium ferricyanide, respectively. Sodium dithion-

ite and potassium ferricyanide were then removed by dialysis against 100 volumes of distilled water for 2 h at 4°C. The purity of the solutions of haemoglobin or methaemoglobin were determined spectrophotometrically, and the solutions were frozen and stored in aliquots at −20°C for up to 14 days.

Preparation of haemoglobin-agarose and methaemoglobin-agarose

Haemoglobin-agarose (Sigma) was supplied entirely in the oxidised form as methaemoglobin-agarose. Glass columns were filled with methaemoglobin-agarose (2.5 × 0.5 cm or in some experiments 3.5 × 0.5 cm) and if required, the methaemoglobin was reduced to haemoglobin by treating the columns with 3 ml of 2×10^{-3} M sodium dithionite. This reduction was accompanied by a characteristic change in colour from brown to red. Columns were then extensively washed with double-distilled water. Solutions (4 ml) of sodium azide (100 μM), glyceryl trinitrate (30 nM and 10 μM) and extracts containing IF were applied to columns of haemoglobin-agarose or methaemoglobin-agarose in a cold room at 4°C but for reasons of speed, solutions of nitric oxide were pumped through columns at a rate of 4 ml min^{-1} at room temperature.

Drugs

Glyceryl trinitrate, 10% w/w in lactose, batch 13, was obtained from Napp Laboratories. Phenylephrine and sodium azide were obtained from Sigma. All drugs were dissolved in twice-distilled water.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Glyceryl trinitrate and sodium azide

Pretreatment of endothelium-denuded rings of rabbit aorta for 20 min with methaemoglobin (10 μM) resulted in an inhibition (1.7 fold parallel shift, to the right, $n = 5$) of the relaxation induced by cumulative additions of glyceryl trinitrate (0.1 nM–1 μM), but a larger inhibition (5.0 fold parallel shift to the right, $n = 5$) of the relaxation induced by sodium azide (1 nM–10 μM) (Figure 1). Haemoglobin pretreatment (10 μM, 20 min) was more effective than methaemoglobin pretreatment (10 μM, 20 min) at blocking the relaxation induced by either glyceryl trinitrate (0.1 nM–10 μM) or azide (1nM–100 μM), and in each case the blockade was accompanied by a depression of the maximum response (Figure 1).

Solutions of glyceryl trinitrate (30 nM and 10 μM,

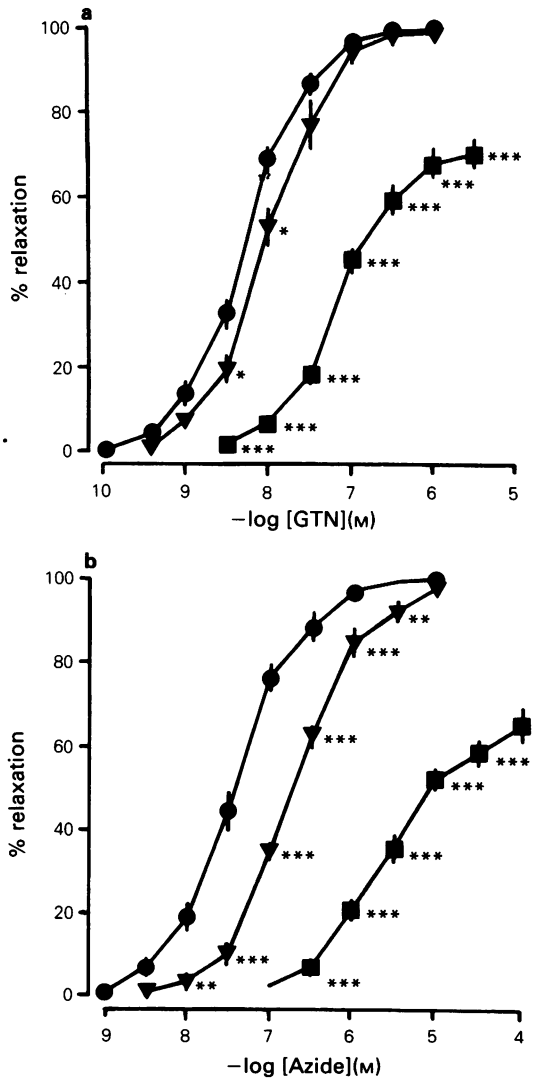

Figure 1 Dose-response curves showing the effects of haemoglobin and methaemoglobin on the relaxations induced by (a) glyceryl trinitrate (GTN) and (b) sodium azide. Control responses (●) and responses to the nitrovasodilators following pretreatment for 20 min with haemoglobin at 10 μM (■) or methaemoglobin at 10 μM (▼) are shown. Each point is the mean of 5–9 observations and vertical lines represent s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

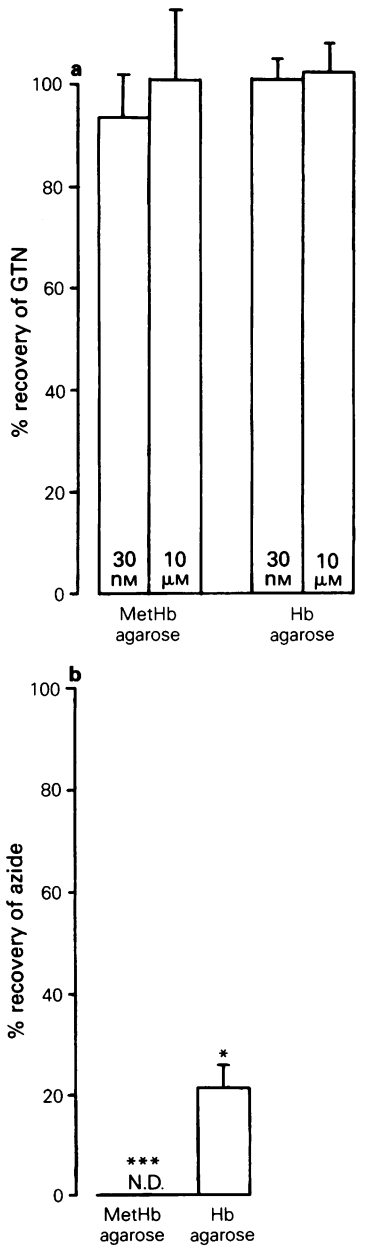

Figure 2 The percentage recovery of vasodilator activity in the eluates following application of 4 ml volumes of 30 nM or 10 μM glyceryl trinitrate (a) or 100 μM sodium azide (b) to 2.5 × 0.5 cm columns of haemoglobin-agarose (Hb-agarose) or methaemoglobin-agarose (Met Hb-agarose), quantified on endothelium-denuded rings of rabbit aorta. Means ± s.e.mean of 4 observations are shown. N.D. indicates none detected. $*P<0.05$, $***P<0.001$, indicate a significant difference from control.

4 ml) passed through 2.5 × 0.5 cm columns of either haemoglobin-agarose or methaemoglobin-agarose, retained their full vasodilator activity (Figure 2), indicating that glyceryl trinitrate had not been bound or inactivated. However, with solutions of azide (100 μM, 4 ml), only 21.3 ± 3.8% ($n = 4$) of the vasodilator activity was recovered in the eluates from haemoglobin-agarose columns and no activity was recovered in the eluates from methaemoglobin-agarose columns (Figure 2). The loss of azide from the eluates was due to binding rather than inactivation, since all of the applied azide could be eluted from the methaemoglobin-agarose columns when displaced by potassium cyanide (200 μM, 4 ml, data not shown), a ligand with a higher affinity than azide for methaemoglobin (Antonini & Brunori, 1971).

Nitric oxide

Solutions of freshly-generated nitric oxide induced rapid transient relaxation of endothelium-denuded rings of rabbit aorta (Figure 3), as previously described (Gruetter *et al.*, 1980). Simultaneous addition of 100 μl aliquots of nitric oxide solution to control aortic rings and to rings pretreated for 20 min with either haemoglobin (1 μM) or methaemoglobin (1 μM) showed that haemoglobin was more effective than methaemoglobin at inhibiting vasodilatation (Figure 3a, $n = 4$). Furthermore, when 4 ml aliquots of nitric oxide solution were applied simultaneously to 2.5 × 0.5 cm columns of haemoglobin-agarose or methaemoglobin-agarose, no activity was recovered in the eluates from haemoglobin-agarose but almost all of the original activity was recovered in the eluates from methaemoglobin-agarose columns (Figure 3b, $n = 4$).

Bovine retractor penis inhibitory factor

Extracts containing the acid-activated form of IF (Gillespie & Martin, 1980) induced rapid, transient relaxation of endothelium-denuded rings of rabbit aorta (Figure 4). Relaxation induced by IF was blocked more readily by pretreatment of aortic rings for 10 min with haemoglobin (3 μM, $n = 4$) than by pretreatment with methaemoglobin (3 μM, $n = 4$), confirming previous findings by Bowman *et al.* (1982).

Aliquots (4 ml) of extracts containing IF in the acid-activated form were passed through 3.5 × 0.5 cm columns of methaemoglobin-agarose ($n = 6$) or haemoglobin-agarose ($n = 13$). All of the original activity was recovered in the eluates from methaemoglobin-agarose columns, and this was stable (on ice). Little or no activity was recovered in the eluates from haemoglobin-agarose columns (Figure 5); reactivation of these eluates at pH 2 for 10 min, followed by neutralization (Gillespie & Martin, 1980), led to a

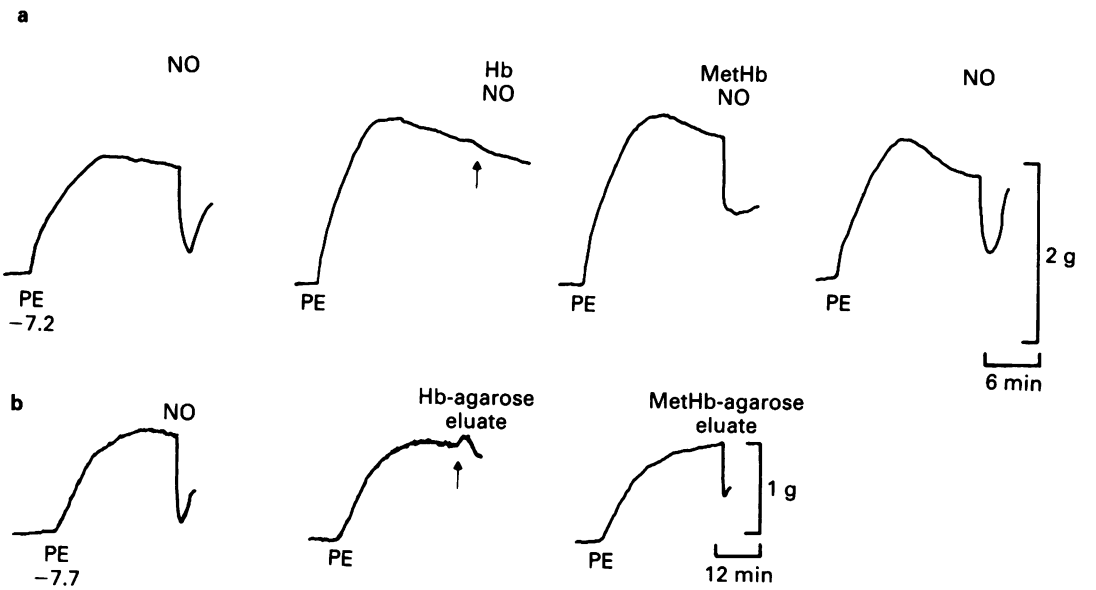

Figure 3 The ability of haemoglobin (Hb) or methaemoglobin (MetHb) to inhibit nitric oxide-induced relaxations of phenylephrine (PE)-contracted endothelium-denuded, rings of rabbit aorta and the removal of nitric oxide from solution following passage through columns of haemoglobin-agarose or methaemoglobin-agarose. (a) Simultaneous additions of 100 μl volumes of nitric oxide solution (NO) to control rings and to rings pretreated for 20 min with Hb (10 μM) or MetHb (10 μM). (b) Simultaneous additions of 100 μl volumes of nitric oxide solution (NO) and of eluates following passage of 4 ml aliquots of nitric oxide solution through 2.5 × 0.5 cm columns of Hb-agarose or MetHb-agarose. Molar concentrations are expressed in log units.

partial recovery of the original vasodilator activity (in 11 out of 13 experiments), but this activity was unstable and decayed rapidly even on ice (Figure 5); activity was not recovered in 2 experiments, nor could it be recovered following elution of columns with distilled water at pH 2, which oxidized the haemoglobin to methaemoglobin. The unstable vasodilator activity in eluates from haemoglobin-agarose columns was, like the original IF, inhibited by pretreating aortic rings with haemoglobin (3 μM, data not shown).

Similar results were obtained with IF in the inactive form. After acid-activation at pH 2, eluates from the methaemoglobin-agarose columns ($n = 5$) contained stable (on ice) activity equal to that of control extracts, whereas eluates from haemoglobin-agarose columns ($n = 5$) contained variable amounts of activity which was unstable on ice (Figure 6), or in two experiments, no activity.

Discussion

In previous studies using rabbit aorta we showed that haemoglobin was a potent inhibitor of the vasodilatation and associated increases in cyclic GMP induced by EDRF and the nitrovasodilator, glyceryl trinitrate (Martin *et al.*, 1985a). Since only ferrous haemoproteins with ligand binding properties shared the inhibitory activity of haemoglobin (Martin *et al.*, 1985b), we proposed that haemoglobin may exert its blocking action by binding the active principles, so

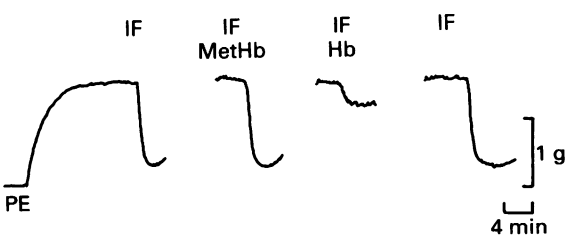

Figure 4 Blockade of the vasodilator action of bovine retractor penis inhibitory factor (IF) in phenylephrine (PE, 0.1 μM)-contracted endothelium-denuded rings of rabbit aorta following 10 min pretreatment with methaemoglobin (3 μM) or haemoglobin (3 μM). The final bath concentration of IF was equivalent to 80 mg of retractor penis muscle per ml.

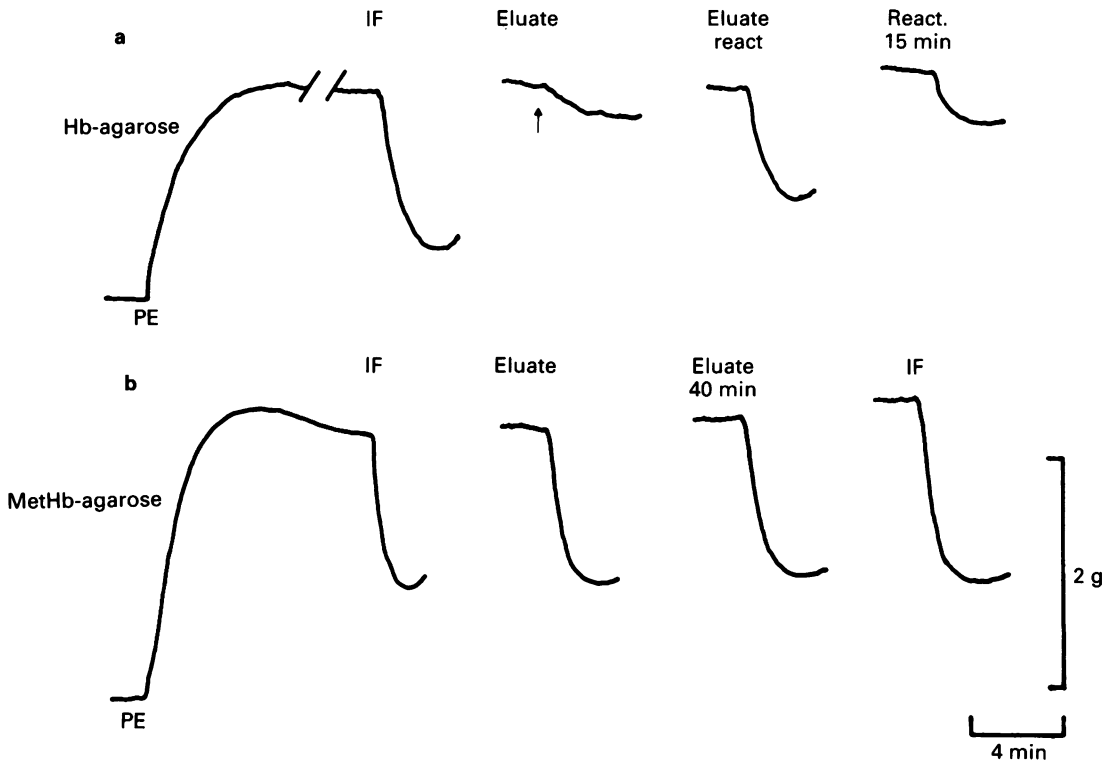

Figure 5 Assay on phenylephrine (PE, 0.1 μM)-contracted endothelium-denuded rings of rabbit aorta of activity in the eluates following application of 4 ml aliquots of extracts, containing inhibitory factor (IF) in the acid-activated form, to 3.5 × 0.5 cm columns of (a) haemoglobin-agarose (Hb-agarose) and (b) methaemoglobin-agarose (MetHb-agarose). The activity in the control extract is shown (IF). Following application to Hb-agarose, little activity was recovered in the eluate (Eluate), but when reactivated at pH 2 for 10 min and tested immediately, more activity was recovered (Eluate react.) although this was unstable and decayed substantially within 15 min (React. 15 min) despite being kept on ice. Following application to MetHb-agarose the activity in the eluate (Eluate) was equal to that of the original extract (IF) and was stable, it had not decayed when tested 40 min later (Eluate 40 min). The final bath concentration of IF was equivalent to 80 mg of retractor penis muscle per ml.

preventing them from interacting with a ferrous haem-containing receptor site on soluble guanylate cyclase (Craven & De Rubertis, 1978; Gerzer *et al.*, 1981a,b; Ohlstein *et al.*, 1982).

We have now extended our observations to another nitrovasodilator, sodium azide. We found that the ferric derivative, methaemoglobin is less effective than haemoglobin at blocking relaxation. Data from experiments in which glyceryl trinitrate and azide were passed through columns of haemoglobin-agarose or methaemoglobin-agarose confirmed that azide is reversibly bound to methaemoglobin and, to a lesser extent, to haemoglobin (Antonini & Brunori, 1971). However, the pattern of relative binding and the absence of binding of glyceryl trinitrate indicated that the inhibitory action of haemoglobin or methaemoglobin on the relaxant effects of glyceryl trinitrate or azide cannot be attributed to the binding of these agents. The ability of methaemoglobin to bind azide but not glyceryl trinitrate may, however, explain why this ferric haemoprotein is more effective at inhibiting vasodilatation induced by azide than that induced by glyceryl trinitrate (Figure 1).

The nitrovasodilators induce vascular relaxation by activating soluble guanylate cyclase (Rapoport *et al.*, 1983). This activation is not direct, but proceeds through the intracellular formation of nitric oxide (Arnold *et al.*, 1977; Katsuki *et al.*, 1977). Nitric oxide binds to the ferrous haem-containing receptor site of soluble guanylate cyclase (Craven & De Rubertis, 1978; Ohlstein *et al.*, 1982; Tsai *et al.*, 1983), and thereby activates the enzyme. Nitric oxide has an extremely high affinity for the ligand binding site of haemoglobin but much lower affinity for that of

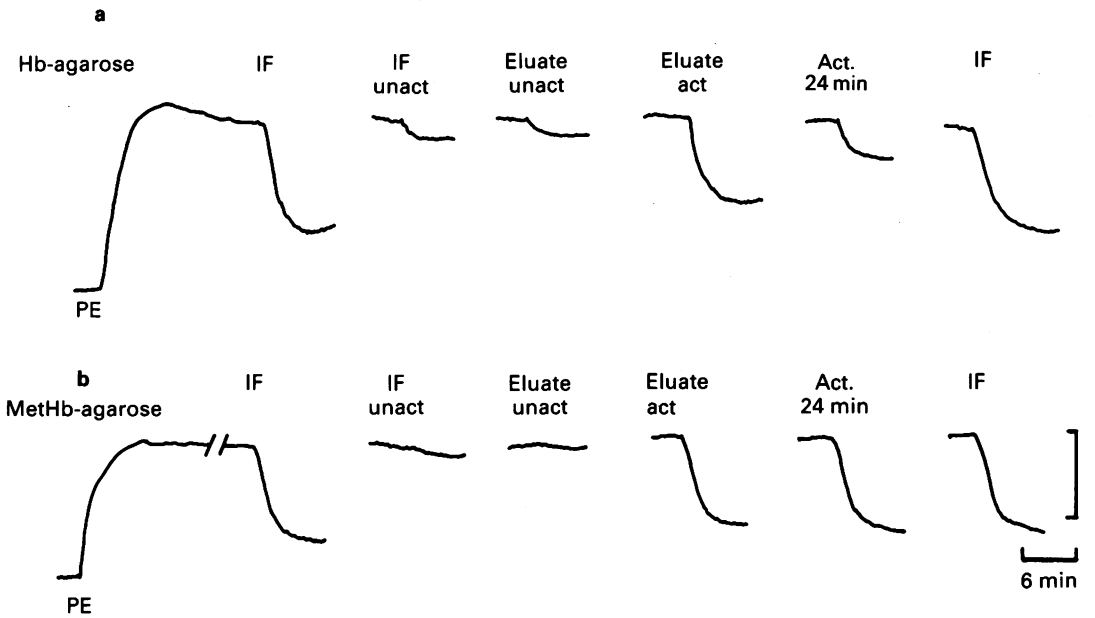

Figure 6 Assay on phenylephrine (PE, 0.1 μM)-contracted endothelium-denuded rings of rabbit aorta of activity in the eluates following the application of 4 ml aliquots of extracts containing inhibitory factor (IF) in the inactive form to 3.5 × 0.5 cm columns of (a) haemoglobin-agarose (Hb-agarose) and (b) methaemoglobin-agarose (MetHb-agarose). The activity in the original extracts before (IF unact.) and after activation at pH 2 (IF) is shown. Following application to a Hb-agarose column no activity was recovered in the eluate (Eluate unact.) until it was activated at pH 2 for 10 min (Eluate act.), but this activity was unstable and had decayed substantially when tested 24 min later (Act 24 min). Following application to MetHb-agarose no activity was found in the eluate before activation at pH 2 (Eluate unact.) but activity equal to the original activity of the extract was recovered following activation (Eluate act.). The activity recovered was stable and had not decayed when tested 24 min later (Act. 24 min). The final bath concentration of IF was equivalent to 40 mg of retractor penis muscle per ml.

methaemoglobin (Gibson & Roughton, 1957; Antonini & Brunori, 1971). In keeping with this, we found that relaxation induced by solutions of freshly-generated nitric oxide was blocked more readily by haemoglobin than by methaemoglobin. Furthermore, nitric oxide was removed from solution more readily when passed through columns of haemoglobin-agarose than columns of methaemoglobin-agarose. Haemoglobin therefore may inhibit vasodilatation induced by glyceryl trinitrate and azide by binding their active principle, nitric oxide. The large molecular size of haemoglobin precludes its entry into the vascular smooth muscle cells, where nitric oxide is presumed to be generated from the parent nitrovasodilator (Arnold *et al.*, 1977; Katsuki *et al.*, 1977). However, it is possible that haemoglobin in the interstitial space might act as a 'sink' for the freely permeable nitric oxide gas, drawing it out of the cells and thereby inhibiting its vasodilator action.

Cocks & Angus (1985), using a cascade bioassay, presented evidence that haemoglobin-agarose bound or inactivated EDRF. We examined the ability of haemoglobin-agarose and methaemoglobin-agarose to interact with an analogous, similarly unidentified substance IF (Ambache *et al.*, 1975; Gillespie & Martin, 1980). IF is isolated from bovine retractor penis muscle in an inactive form but can be converted to the active form by brief exposure to acid at pH 2 (Gillespie & Martin, 1980). It is a similar if not identical substance to EDRF. Both IF and EDRF are labile, anionic, hydrophilic, borohydride-sensitive substances (Ambache *et al.*, 1975; Gillespie & Martin, 1980; Gillespie *et al.*, 1981; Griffith *et al.*, 1984; Cocks & Angus, 1985; Cocks *et al.*, 1985), whose ability to elevate cyclic GMP levels and induce smooth muscle relaxation is blocked by haemoglobin but not by methaemoglobin (Bowman *et al.*, 1981; 1982; Bowman & Drummond, 1984; Martin *et al.*, 1985a,b).

We found that acid-activated IF was unaffected by passage through columns of methaemoglobin-agarose, but that its relaxant activity was lost by passage through haemoglobin-agarose. IF appeared

not to have been retained by haemoglobin-agarose, since activity could not be recovered from these columns by oxidizing haemoglobin to methaemoglobin, which does not interact with IF. The inactive eluates could, however, be reactivated by acid (pH 2) (Gillespie & Martin, 1980), though recovery was only partial and the activity had become unstable and decayed rapidly even on ice. Similar results were obtained with the inactive form of IF. Acid-activated eluates from methaemoglobin-agarose columns contained the original, stable activity but eluates from haemoglobin-agarose columns contained variable activity that decayed rapidly on ice. It is possible therefore, that haemoglobin might inhibit the vasodilatation induced by IF not by binding and retaining the substance, but by interacting with it and rendering it unstable. Although the mechanism by which haemoglobin inactivates IF is obscure, the finding that the oxidized form of haemoglobin, methaemoglobin, is much less effective might indicate the involvement of a redox-type reaction.

Although Cocks & Angus (1985) suggested that haemoglobin-agarose could bind EDRF, their experiments did not differentiate between binding and chemical inactivation. Since IF is similar if not identical to EDRF it is tempting to speculate that the ability of haemoglobin to block the actions of EDRF (Martin *et al.*, 1985a) might reflect chemical inactivation rather than irreversible binding of this vasodilator.

In conclusion, blockade by haemoglobin of the vasodilatation of rabbit aorta induced by glyceryl trinitrate and by azide does not appear primarily to involve a direct interaction between haemoglobin and the nitrovasodilators. It may, however, reflect binding of their active principle, nitric oxide. Blockade of the vasodilator actions of IF, and possibly also of EDRF, appears to involve chemical inactivation by haemoglobin rather than irreversible binding by the haemoprotein.

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