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Evidence that inhibitory factor extracted from bovine retractor penis is nitrite, whose acid-activated derivative is stabilized nitric oxide

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1 Unactivated extracts of bovine retractor penis (BRP) contain 3–7 μM nitrite. Acid-activation of these extracts at pH 2 for 10 min followed by neutralization generates the active form of inhibitory factor (IF; assayed by its vasodilator action on rabbit aorta), and is associated with partial loss of nitrite.

2 Increasing the time of acid-activation at pH 2 from 10 to 60 min with intermittent vortex mixing generates greater vasodilator activity and increases nitrite loss.

3 When acid-activated and neutralized extracts are incubated at 37°C or 30 min or boiled for 5 min, vasodilator activity is lost and nitrite content increased. Reactivation of these samples at pH 2 for 10 min followed by neutralization leads to partial recoveries of vasodilator activity with loss in nitrite content.

4 Addition of sodium nitrite to BRP extracts increases acid-activatable vasodilator activity *pro rata*.

5 Acid-activation of aqueous sodium nitrite solutions results in less loss of nitrite and generation of less vasodilator activity than BRP extracts. Vasodilatation is only transient and is rapidly abolished on neutralization, whereas responses to acid-activated BRP extracts are more prolonged and activity is stable on ice.

6 Bovine aortic endothelial cells yield vasodilator activity that is indistinguishable from that isolated from BRP. It is activated by acid, stable on ice, abolished by boiling or by haemoglobin, and appears to be due to the generation of nitric oxide (NO) from nitrite.

7 The data provide confirmatory evidence that nitrite in BRP extracts is IF, that acid-activation of BRP extracts yields NO which is responsible for its vasodilator action, and that inactivation occurs by decay of NO to nitrite and nitrate. They further suggest that BRP extracts contain a NO-stabilizing agent which favours conversion of nitrite to NO.

8 The finding that bovine aortic endothelial cells yield an agent indistinguishable from IF suggests that nitrite in endothelial cells may likewise be the precursor of endothelium-derived relaxing factor (EDRF), itself identified as NO.

Introduction

In the search for the unidentified neurotransmitter of the bovine retractor penis (BRP) muscle, an inhibitory factor (IF) was isolated which mimicked the action of the inhibitory nerve (Ambache *et al.*, 1975; Gillespie & Martin, 1980). IF has subsequently been found to have many properties in common with endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). Both IF and EDRF are extremely labile, anionic, hydrophilic, borohydride-sensitive substances (Gillespie & Martin, 1980; Gillespie *et al.*, 1981; Cocks & Angus, 1985; Cocks *et al.*, 1985; Griffith *et al.*, 1984). Furthermore, the ability of both IF and EDRF to induce smooth muscle relaxation and elevate cyclic GMP levels is blocked by two agents that inhibit the stimulation of soluble guanylate cyclase, haemoglobin and methylene blue, and potentiated by the selective inhibitor of cyclic GMP phosphodiesterase, M&B 22,948 (Bowman *et al.*, 1982; Bowman & Drummond, 1984; Martin *et al.*, 1985; 1986a; Griffith

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et al., 1985). IF is extracted from BRP in an inactive form but is converted to the active form by brief exposure to acid (Gillespie & Martin, 1980). Murray et al. (1986) have demonstrated that an EDRF-like substance released from bovine aortic endothelial cells is similarly activated by acid. Recent studies now indicate that EDRF is nitric oxide (NO) (Furchgott, 1987; Palmer et al., 1987; Ignarro et al., 1987). Furchgott (1987) has postulated that the inactive form of IF is inorganic nitrite and the active form, generated by acid, is NO. We have further investigated whether NO can account for the vasodilator activity of IF derived from BRP. In addition, we have investigated whether IF can be extracted from bovine aortic endothelial cells.

Methods

Organ bath studies

IF was bioassayed on rabbit aortic rings as previously described (Martin et al., 1986b). Briefly, male New Zealand White rabbits weighing 2–3 kg were killed by stunning and exsanguination. The aorta was removed, cleared of adhering fat and cut into transverse rings 2.5 mm wide. Endothelial cells were removed by gently rubbing the intimal surface with a moist wooden stick for 30 s. Successful removal of endothelial cells was confirmed later by the inability of acetylcholine (1 μM) to induce relaxation. The aortic rings were mounted under 2 g resting tension on stainless steel hooks in 5 ml organ baths and bathed at 37°C in Krebs solution containing (mM): NaCl 119, 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 with Ormed UF1 force transducers and displayed on an Ormed Multitrace 8 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.

The vasodilator activity of IF was assayed against submaximal (40–60% of maximum) tone induced by phenylephrine (PE). In all experiments the vasodilator activity of extracts was measured by plotting the log of the volume of extract against the % relaxation of PE-induced tone. The fold-difference in vasodilator activity of test samples compared with standard extract was obtained by calculating the ratio of the volumes of standard extract and of test sample which gave identical vasodilator responses.

Preparation of inhibitory factor from bovine retractor penis

Extracts containing IF were prepared as previously described (Gillespie et al., 1981). Briefly, bovine retractor penis muscles obtained from a local abattoir were chopped and extracted overnight with continuous stirring at 4°C in methanol (5 ml g^{-1} tissue). The extract was then filtered and 60 ml aliquots applied to 3.5 × 0.5 cm columns of Bio-Rad AG1-X8 (formate form). The columns were washed with 2 × 5 ml of double-distilled water and eluted with 6 ml of 300 mM NaCl. Eluates were combined, adjusted to pH 9–9.5, passed through an 8.5 × 2.5 cm column of alumina to remove adenine nucleotides (Bowman et al., 1979) and the resulting eluate was neutralized and stored frozen in aliquots at −20°C. Extracts had a final concentration equivalent to 1.6 g of tissue per ml. When required, extracts were acid-activated by adjusting the pH to pH 2 for 10 min with HCl followed by neutralization with NaOH. Extracts were stored on ice during use. In some experiments the number of cells extracted was determined by a fluorescence assay for DNA (Kissane & Robins, 1958), taking 6.3 μg of DNA to be equivalent to 10^6 cells (Davidson, 1976).

Measurement of nitrite

The nitrite content of extracts was measured by diazo formation according to the method of Bennett et al. (1986). Briefly, at 4°C, 0.3 ml samples of extract were mixed with 0.3 ml of 1% (w/v) sulfanilic acid (Sigma) in 2 M HCl. After 5 min, 0.3 ml of 1% (w/v) aqueous N-(1-napthyl) ethylenediamine dihydrochloride (Sigma) was added, and the absorbance of the pink complex was determined at 548 nm. A standard curve was prepared with solutions of sodium nitrite (0.3–30 μM) (Sigma).

Isolation and extraction of bovine aortic endothelial cells

Endothelial cells were isolated from bovine aortae either by scraping the intimal surface with a scalpel blade (Ryan et al., 1980) or by collagenase (0.2%) treatment (Gordon & Martin, 1983). Endothelial cells were either extracted immediately with methanol at 4°C or were grown in culture until confluent and then extracted with methanol at 4°C. Extracts were then prepared as described above for BRP. The number of cells extracted was determined by DNA analysis (Kissane & Robins, 1958).

Drugs

Phenylephrine and haemoglobin (bovine type 1) were obtained from Sigma. Haemoglobin was reduced before use by dithionite treatment (Martin et al., 1985).

Statistical analysis

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

Results

Vasodilator activity and nitrite content of extracts of BRP

Unactivated extracts of BRP muscle had little vasodilator activity when assayed on phenylephrine (PE)-contracted rings of rabbit aorta. They contained 3.9 ± 0.1 μM nitrite (n = 6) (Figure 1). When aliquots of extract were acid-activated, i.e. taken to pH 2 for 10 min and neutralized, vasodilator activity was generated in association with 74.2 ± 1.5% (n = 6) loss in nitrite content. When incubated at 37°C for 30 min or placed in a boiling water bath for 5 min, acid-activated and neutralized extracts lost most of their vasodilator activity (to 15.7 ± 2.8 and 13.3 ± 3.1%) in association with increases in nitrite content of 58.4 ± 1.2% and 87.1 ± 7.8% (n = 6), respectively. Reactivation (pH 2 for 10 min followed by neutralization) of aliquots of acid-activated and neutralized extract that had been incubated at 37°C or boiled led to recoveries of 56.3 ± 4.1% and 46.0 ± 3.4% (n = 6) of the original vasodilator activity, and losses in nitrite content of 60.0 ± 3.8% and 78.3 ± 3.8%, respectively (Figure 1).

Effects of prolonged exposure at pH 2

In a separate experiment, aliquots of unactivated extract containing nitrite at a concentration of 3.3 ± 0.1 μM (n = 6) were acid-activated at pH 2 either for 10 min and neutralized, or for 60 min (with 1 min vortex mixing every 5 min) and neutralized (Figure 2). The more prolonged acid-activation led to significantly greater vasodilator activity (78.6 ± 5.3%, $P < 0.001$) and loss in nitrite content (91.0 ± 9.0 cf. 57.5 ± 2.0%, n = 6, $P < 0.001$). Following the prolonged acid-activation, incubation at 37°C for 30 min led to loss of vasodilator activity in association with an increase in nitrite content of 236.7 ± 14.1% (n = 6). Reactivation (pH 2 for 10 min followed by neutralization) then led to a recovery of 62.2 ± 5.9% (n = 6) of the original vasodilator activity and loss in nitrite content of 63.4 ± 13.5% (n = 6) (Figure 2).

Vasodilator activity of acidified nitrite

Aqueous solutions of sodium nitrite (50 μM), acidified to pH 2 using HCl, induced rapid, transient relaxations of rabbit aorta (Figure 3). When neutralized and assayed immediately, no vasodilator activity was observed. Similar volumes of distilled water acidified to pH 2 had no vasodilator activity. Acid-activated and neutralized extracts of BRP (which contained 3.6 ± 0.1 μM nitrite before acid-activation) induced larger and more sustained relaxations than those induced by acidified nitrite (50 μM) (Figure 3)

Loss of nitrite with acid-activation compared in BRP extracts and aqueous nitrite solutions

Following acidification (pH 2 for 10 min) and neutralization, the loss of nitrite from BRP extracts containing 6.4 ± 0.2 μM nitrite was significantly greater than from aqueous solutions of 9.8 ± 0.2 μM sodium nitrite (34.2 ± 1.2% cf. 8.8 ± 0.1%, n = 5, $P < 0.001$) (Figure 4). Following more prolonged acid-activation (pH 2 with 1 min vortex mixing every

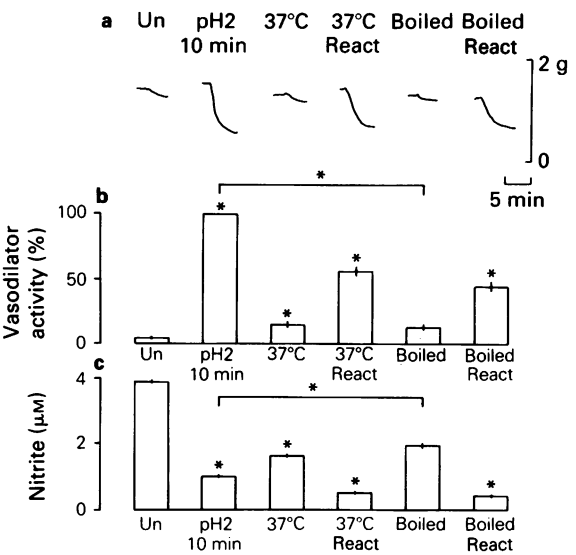

Figure 1 Reciprocal changes in vasodilator activity and nitrite content of extracts of bovine retractor penis (BRP). Vasodilator activity was measured on phenylephrine-contracted rings of rabbit aorta and nitrite content was measured by diazo formation in unactivated (Un), acid-activated and neutralised (pH 2 10 min) and acid-activated and neutralised samples that were incubated at 37°C for 30 min (37°C) and subsequently reactivated by acid (37°C React) or boiled for 5 min (Boiled) and subsequently reactivated by acid (Boiled React). In (a) is shown the tension recording obtained in one experiment and (b) and (c) give the mean of vasodilator activity and nitrite content, respectively, obtained from 6 experiments; vertical lines show s.e.mean. *$P < 0.001$ indicates a significant difference from the column on the left or a difference between columns joined by a bar.

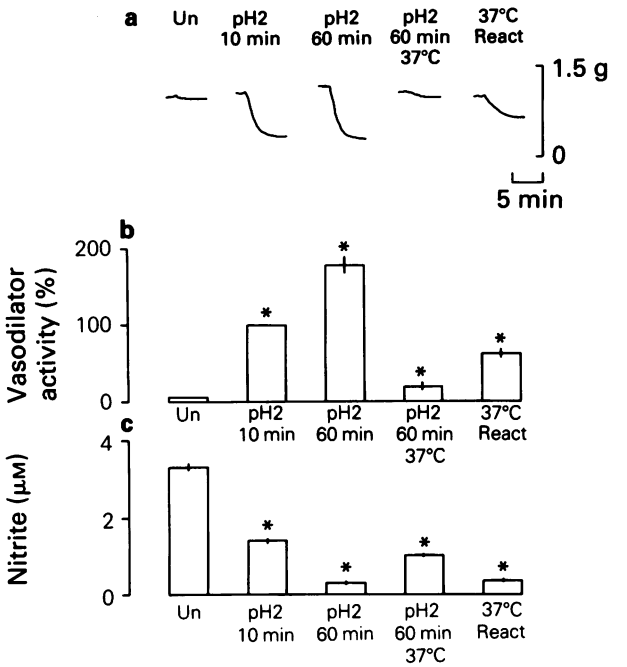

Figure 2 Reciprocal change in vasodilator activity and nitrite content of extract of bovine retractor penis (BRP) taken to pH 2 for 10 min or 60 min. Vasodilator activity was measured on phenylephrine contracted rings of rabbit aorta and nitrite content was measured by diazo formation in samples that were unactivated (Un), acid-activated for 10 min and neutralised (pH 2 10 min), or taken pH 2 for 60 min with intermittent vortexing followed by neutralisation (pH 2 60 min) and then incubated at 37°C for 30 min (pH 2 60 min 37°C) and subsequently reactivated by acid (37°C React). In (a) is shown the tension recording obtained in one experiment and (b) and (c) give the mean of the vasodilator activity and nitrite content respectively, obtained from 6 experiments; vertical lines show s.e.mean. $*P < 0.001$ indicates a significant difference from the column on the left.

5 min for 60 min) and neutralization, the loss of nitrite from BRP extracts was again significantly greater than from aqueous nitrite solutions (92.4 ± 9.4%, $n = 8$ cf. 17.3 ± 0.2%, $n = 4$, $P < 0.001$). (Figure 4).

Effect of adding further nitrite to extracts of BRP

The nitrite content of unactivated extracts of BRP was increased by 3–50 μM by the addition of aqueous sodium nitrite. These unactivated samples had no vasodilator activity. Acid-activation (pH 2 for 10 min followed by neutralisation) led to the generation of vasodilator activity (Figure 5). This vasodilator activity, like that derived from the original extract, was abolished by boiling and (unlike that derived from acidified and neutralised aqueous solutions of nitrite), stable on ice. A direct linear relationship was observed between added nitrite and fold increase in vasodilator activity (Figure 5). Furthermore, an estimate of the original nitrite content of the extract, obtained from the intercept on the concentration axis (3.8 ± 0.7 μM, $n = 8$), is close to the actual value of 3.6 ± 0.1 μM measured by diazo formation.

Extraction of inhibitory factor from bovine aortic endothelial cells

Extracts of bovine aortic endothelial cells had vasodilator activity after acid-activation (Figure 6). Like that of BRP extracts, this was abolished by boiling or by pretreating the rabbit aortic rings for 10 min with haemoglobin (10 μM) (Figure 6). The vasodilator activity of extracts of bovine aortic endothelial cells was 85 ± 31% ($n = 6$) of that obtained from an equivalent number of cells of BRP, though the nitrite content was 7 times higher: extracts of bovine aortic endothelial cells contained 136 ± 28 pmol per 10^6 cells ($n = 4$) whereas extracts of BRP contained 19 ± 2 pmol per 10^6 cells ($n = 6$).

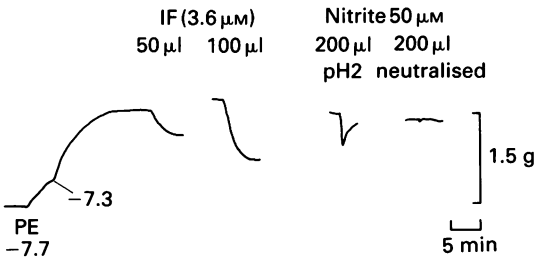

Figure 3 Comparison on a phenylephrine (PE)-contracted ring of rabbit aorta of the vasodilator activity of an acid-activated extract of bovine retractor penis and that of an acidified aqueous solution of nitrite. The responses to 50 μl and 100 μl of acid-activated and neutralised extract containing 3.6 μM nitrite (IF) are shown along with responses to 200 μl of a solution of 50 μM nitrite acidified to pH 2 and to 200 μl of the acidified solution of nitrite immediately upon neutralisation.

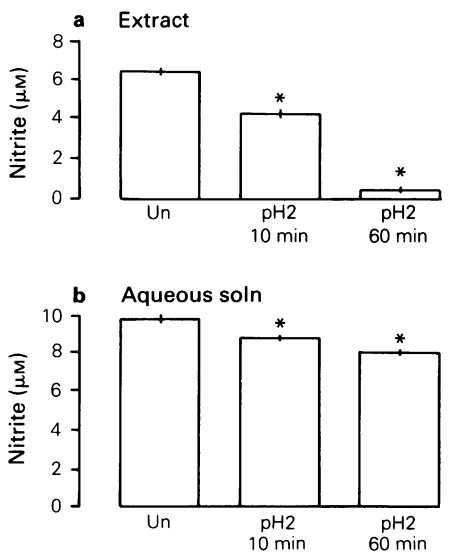

Figure 4 Comparison of the loss of nitrite from extracts of bovine retractor penis (BRP) and from aqueous nitrite solutions at pH 2. The nitrite content of extracts of BRP (a) and of aqueous solutions of sodium nitrite (b) were measured before acidification (Un), after being taken to pH 2 for 10 min and subsequently neutralised (pH 2 10 min) and after being taken to pH 2 for 60 min with intermittent vortexing followed by neutralisation (pH 2 60 min). Results are presented as the mean obtained from 4–8 experiments; vertical lines show s.e. mean.

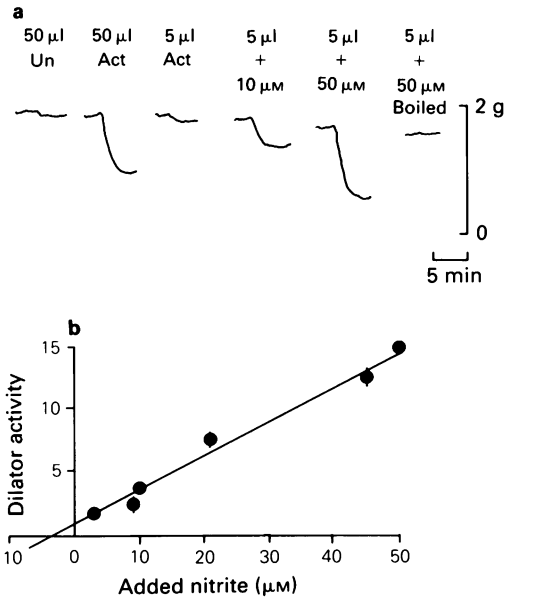

Figure 5 Addition of further nitrite to extracts of bovine retractor penis before acid-activation leads to proportionate increases in vasodilator activity. In (a) is shown the vasodilator activity of 50 μl of unactivated extract (Un), 5 μl and 50 μl of acid-activated extract (Act) and 5 μl of extract to which 10 μM or 50 μM nitrite was added before acid-activation. The abolition by boiling of the vasodilator response to the extract to which 50 μM nitrite had been added is also shown. A graph of the fold increase in vasodilator activity when compared with standard extract versus the concentration of nitrite added is shown in (b). The line through the points was calculated by least-squares analysis. Each point is the mean of 8 observations; vertical lines show s.e.mean.

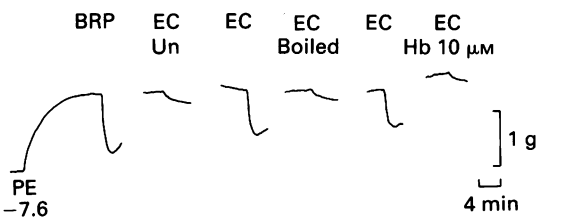

Figure 6 Assay on phenylephrine (PE)-contracted ring of rabbit aorta showing the vasodilator activity of acid-activated extracts of bovine retractor penis (BRP) and of extracts of bovine aortic endothelial cells that were unactivated (EC Un), acid-activated (EC), acid activated and boiled for 5 min (EC Boiled) and acid-activated and tested following pretreatment of the aortic ring for 10 min with haemoglobin at 10 μM (EC Hb 10 μM).

Discussion

Nitrous acid, formed on acidification of nitrite, leads to the generation of NO which subsequently decays in the presence of oxygen and water to form equal amounts of nitrous and nitric acids (Sisler, 1956):

$$2HNO_2 \rightleftharpoons NO + NO_2 + H_2O \quad (1)$$
$$2NO + O_2 \rightarrow 2NO_2 \quad (2)$$
$$2NO_2 \rightleftharpoons N_2O_4 \quad (3)$$
$$N_2O_4 + H_2O \rightleftharpoons HNO_2 + HNO_3 \quad (4)$$

Upon neutralisation the strong nitrous and nitric acids dissociate giving rise to free nitrite and nitrate, respectively.

We found that unactivated extracts of BRP contain nitrite in micromolar concentrations and that acid-activation, which generates the active form of IF (Gillespie & Martin, 1980), is associated with a loss of nitrite. This is consistent with the proposal of Furchgott (1987) that the inactive form of IF is inorganic nitrite and the active form NO. Taking extracts to pH2 for 60 min with intermittent vortexing to remove free NO led to an even greater increase in vasodilator activity and associated decline in nitrite content, in accordance with driving equation (1) further to the right. Furthermore, the loss of vasodilator activity of the active form of IF on incubation at 37°C or boiling, was associated with an increase in nitrite content; reactivation of these samples, which led to partial recovery of the original vasodilator activity, was associated with a further loss of nitrite. These observations can be explained by the decay of NO to nitrite and nitrate according to equation (4) at 37°C or following boiling, and the renewed generation of NO from the remaining nitrite upon reactivation. Following incubation at 37°C or boiling, the recovery of nitrite lost during the preceding acid-activation was less than the 50% expected from equation (4), probably because NO and NO_2 escape into the atmosphere.

We confirmed the observation of Furchgott (1987) that acidification of nitrite leads to an enhancement of vasodilator activity. NO is a powerful vasodilator (Gruetter *et al.*, 1980, Martin *et al.*, 1986b), but none of the other products of the reactions, i.e. nitrogen dioxide (NO_2) or nitrate have any vasodilator activity and nitrite has only weak activity. Acidified aqueous solutions of nitrite had, however, less vasodilator activity than BRP extracts and its action was faster in onset and more transient. Moreover, the vasodilator activity of acidified nitrite was lost immediately upon neutralisation, consistent with the decay of NO to NO_2, whereas the activity of IF was stable after neutralisation if stored on ice.

These apparent differences between nitrite and IF could be explained, however, if the extracts contained an additional substance or substances which reversibly bound and stabilised the NO formed during acidification, thereby effectively taking NO out of solution and driving the equilibrium of equation (1) further to the right. Acidification did induce greater loss of nitrite from BRP extracts than from aqueous nitrite solutions. The greater and more prolonged vasodilator activity of BRP extracts than of acidified aqueous solutions of nitrite is also consistent with the presence of a binding substance stabilizing NO. The most convincing evidence for the existence of a binding and stabilizing substance for NO in BRP extracts comes, however, from experiments in which additional nitrite was added to extracts prior to acid-activation. The vasodilator activity of these extracts was enhanced in direct proportion to the fold-increase in nitrite content. This vasodilator activity was, like that of the original extract but unlike that of acidified and neutralised nitrite, stable on ice.

The nature of the putative stabilizing substance for NO in extracts of BRP is not yet known, but proteins, thiols and sugars are known to bind NO, potentiating and prolonging its ability to activate soluble guanylate cyclase (Braughler *et al.*, 1979). The stabilizing substance is probably negatively charged since during the preparation of extracts it bound to and eluted from anion columns.

The identity of IF accounts for many of its known properties. It explains why IF is hydrophilic and binds in both the active and inactive form to anion exchange resin (Gillespie *et al.*, 1981); it explains the ability of the active form to elevate cyclic GMP levels (Bowman & Drummond, 1984) through activation of soluble guanlyate cyclase by NO (Arnold *et al.*, 1977); and it explains how the actions of IF are blocked by haemoglobin (Bowman *et al.*, 1982; Bowman & Drummond, 1984), whose ligand binding site has an exceptionally high affinity for NO (Gibson & Roughton, 1957).

We originally found, that at room temperature or at 37°C the active form of IF reverts back to the inactive form which can subsequently be reactivated (Gillespie & Martin, 1980). Equation (4) predicts that only 50% of the NO generated can revert back to nitrite and the experimental results show that the reversion was even less than 50%. As a consequence of decay to nitrate and loss of NO and NO_2 to the atmosphere, less nitrite is available for the generation of NO during sequential acid-activations; this is reflected in the decline in vasodilator activity observed experimentally. In addition, we orginally reported that boiling extracts led to loss of activity that could not be regenerated upon reactivation (Gillespie & Martin, 1980). We now find that the vasodilator activity of extracts can be partially restored upon reactivation after boiling, as after incubation at 37°C. Whether the difference between our previous and present results arise from differences in extraction procedure or improvements in quantitative analysis is not clear.

The similarities between EDRF and the active form of IF are explained if both are NO (Furchgott, 1987; Palmer *et al.*, 1987; Ignarro *et al.*, 1987). We found that vasodilator activity indistinguishable from that of IF could be extracted also from bovine aortic endothelial cells, raising the possibility that the endothelial precursor of EDRF could be nitrite. When the number of cells extracted was taken into consideration, endothelial extracts had approximately equal vasodilator activity to BRP extract, but contained about 7 times more nitrite. The explanation of why the increased nitrite content of endothelial extracts was not reflected in increased vasodilator activity might possibly reflect differences in the abilities of endothelial and BRP extracts to stabilize NO. It is inconceivable that acid-activation at pH 2 is a physiological means of generating NO. Nitrite reductases exist in nature (Ingledew & Poole, 1984), and the possibility that such enzymes are utilized by endothelial cells to produce NO warrants further investigation.

In conclusion, our data show that the inactive form of IF is nitrite and that the acid-activated form is NO, the stability of which is increased by an unidentified anionic component of the extract. EDRF and the active form of IF appear to be identical substances, i.e. NO. Whether nitrite is utilised by endothelial cells and by non-adrenergic, non-cholinergic nerves in the formation of EDRF and inhibitory neurotransmitter, respectively, remains to be determined.

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